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HAMILTON B. G. ROBINSON

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## THE EDITOR'S VIEWPOINT

Adler, Kesztyüs, and Simon (p. 713) approached the problem of procaine allergy by using precipitation inhibition tests to determine whether group specificity existed among local anesthetics. On the basis of this test, group specificity was exhibited with certain esterified derivatives of PABA. This experimental approach indicates the pathway to be followed to avoid the effects of procaine allergy. Much more time has been necessary to carry this investigation to the present level of accomplishment on the basis of observation rather than experiment.

Investigation of the oral flora in health or in disease has been attempted and abandoned by more than one group. Rosebury, MacDonald, and Clark (p. 718) have undertaken a comprehensive bacteriologic survey of one phase using gingival scrapings from periodontal infections. Direct examinations, animal inoculations, and anaerobic cultivations were utilized. The flora in these conditions of disease was demonstrated to be simply an overgrowth of that found normally. Fusospirochetal infection appeared to be an etiologic factor in Vincent's infection, periodontal disease, and even in the virus disease, herpetic gingivostomatitis. Similar groups of organism were found in different gingival and periodontal diseases.

Mitchell (p. 732) was able to observe two types of periodontal disease in hamsters, one in animals fed coarse diets and with wood shavings available, and the other in animals fed a fine carbohydrate diet. The first type may be comparable to periodontal disease associated with overhanging margins of dental restorations and food impaction in man, while the latter is more suggestive of periodontal disease in man associated with poor oral hygiene. That periodontal destructions and caries both appear associated with the same dentobacterial plaque is of more than passing interest.

Bailey and Phillips (p. 740) have initiated an investigation of the effects of prophylactic technics and materials on enamel. It is surprising that little work has been carried out in this field. They found that ordinary prophylactic preparations reduced enamel luster and scratched enamel, while air-brasive produced greater enamel loss with pitting and loss of luster. However, very brief polishing with alumina restored the original luster. These findings suggest that using pumice or the Airdent on enamel and not following with use of a polishing agent may leave the surface in an undesirable condition.

Sweeney and Taylor (p. 749) have devised a method for determining dimensional changes in dental stone and plaster. The method which they report has great sensitivity and overcomes many of the objections to previous methods. The investigation of dental materials has advanced greatly since the cooperation between the National Bureau of Standards and the American Dental Association was begun. In addition, work, such as that reported here, is being carried on in universities throughout the country in collaboration with or independent of the work at the National Bureau of Standards.

The technic of using zinc chloride and potassium ferrocyanide to reduce dental caries has been investigated by Pelton and a group of cooperators in the State of Wyoming (p. 756). Their results, which were carried out in acceptable fashion, indicate that the claims for the so-called impregnation technic are not valid. It is important that investigations of this type be carried out by several investigators before a method is given complete acceptance. The results given here by Pelton, and in the *J. A. D. A.* 41: 437, 1950, by Ast, Bushel, and Chase, corroborate each other and throw the burden of proof on those who advocate the "impregnation technic."

Koser and Fisher (p. 760) studied the vitamin requirements of oral lactobacilli by developing a basal medium to which factors were added in various combinations. Nico-

tinic acid, pantothenic acid, and biotin are necessary for lactobacillus growth. The other vitamin needs varied from strain to strain. It is suggested that not only may some bacterial strains synthesize certain vitamin factors but that they may be able to substitute other substances to assume the roles of some vitamin in their metabolism. This basic work is necessary to aid in classification of oral lactobacilli and in the broad problem of understanding the oral flora.

Dreizen, Reed, and Spies (p. 774) found that the amino acid-rich salivary mucin is a poor source of essential amino acids for the Hadley strain of lactobacilli in a culture media. However, when an exogenous source of proteolytic or amylolytic enzymes was supplied, salivary mucin served as an efficient source of amino acids. The enzyme systems of the complex colony known as the dentobacterial plaque should be more thoroughly understood. The effects of various factors on certain elements of the plaque may contribute important basic facts but the effects on the total microbial complex that is the plaque must also be considered.

Kirch, Kesel, O'Donnell, and Wach (p. 779) analyzed the salivas of a group of women on an experimental low protein diet. They were able to demonstrate the presence of the eight amino acids for which tests were made, even on very low protein intake. The values for different acids varied to different degrees in the course of the study. The utilization of the salivas of subjects on such experimental diets may afford much useful information.

Scrivener, Myers, Moore, and Warner (p. 784) report on preliminary studies of bacterial antagonism as a possible factor in dental caries immunity. Refinement of their bacteriologic technics should offer useful information. It would be more applicable to test the effectiveness of the organisms in preventing acidogenesis, using enamel rather than dentin fragments, as the decalcification by acids is recognized as beginning in enamel. Bacterial antagonism deserves careful investigation, for it offers possible explanation of the formation of calculus, maintenance of dentobacterial plaques, and the etiology of caries and gingivitis.

Belding and Edwards (p. 791) have grown their subcultured "mucoid variant" of *Str. salivarius* on suitable media and have found that it will digest milk under certain in vitro conditions. A tooth placed in a milk-$CaCo_3$ media and inoculated with *S. odontolyticus* showed a peculiar destruction which apparently progressed between the enamel and dentin. Belding and Edwards interpret this as a possible phase of typical caries.

In spite of some in vitro evidence that nitrofurans inhibit lactobacillus growth, Hufstader, Anderson, Phatak, and Snyder (p. 794) were unable to demonstrate effective reduction of salivary lactobacillus counts with a nitrofuran incorporated in a chewing gum. While this experiment was tried on a small group, another independent study (Ennever, Robinson, and Kitchin, unpublished data) gave equally negative results on a small group who used nitrofuran in a dentifrice.

Shaw (p. 798) studies the effect of gonadectomy on dental caries activity in caries-susceptible white rats and in cotton rats fed a highly purified caries-producing diet. There was a consistent tendency toward lesser average caries incidence in the gonadectomized animals than in their intact littermates. Reduction in dental caries was appreciably higher among males than among females where gonadectomy had been performed but there was no difference in the average dental caries activity in intact males and females. The mechanism by which this change in caries activity takes place is not explained, but in view of previous findings by Hodge (*J. D. Res.* **22**: 275, 1943) on older albino rats and the sex difference noted by some authors in hamsters, further study of the effect of the gonads on dental caries is indicated.

Shaw (p. 802) found that caries-susceptible female white rats subjected to pregnancies and maintained on a highly purified caries-producing diet had a lower average number of carious lesions and significantly lower average tooth decay than in littermates that had never been pregnant and were maintained on the same diet. This finding is in accord with the clinical observations of Ziskin and Hotelling, who found lower caries incidence of dental caries among women who had one or more children than among those who had had none. The mechanism by which pregnancy might protect against dental caries is worthy of the consideration suggested by Shaw. This

laboratory demonstration bears out the clinical finding that the old saying "a tooth for every child" is simply an oft-quoted myth.

Schlack, Gerende, Berzinskas, Mullins, and Taylor (p. 806) were prompted by the results of indirect study to investigate the effect of moisture in the diet on dental caries incidence. The quantity of distilled water consumed by a group of white rats had no effect on dental caries incidence.

On the basis of histologic study of the teeth of 50 albino rats on a Hoppert, Webber, and Canniff diet, Van Huysen (p. 809) concludes that caries is initiated in the albino rat before fracturing occurs. Because decalcification and cavitation precedes fracturing in human caries, Van Huysen suggests that caries in the rat is true caries. Since dental caries in man has not yet been adequately classified, but is a broad term, it does not appear that caries in the rat and in man can be considered identical until more is known of both human and animal caries. Van Huysen's conclusion, however, is that the carious process precedes fracture in the rat and in man.

Losee, Neidig, and Hess, working on a cooperative problem at Georgetown University and the Department of the Navy, report herewith (p. 815) the ratios of histidine, lysine, and arginine in enamel protein. Their results are in close agreement with the ratios for a eukeratin. As these basic studies are continued it is hoped that a clearer picture of the entire organic structure of the dental apparatus will be available. Basic studies on the biochemistry of the teeth in a large part have been directed toward the inorganic content. However, recent studies at a number of laboratories have uncovered valuable information on the organic constituents of tooth protein.

Ring and Levy (p. 817) investigated the alkaline phosphatase activity of the oral epithelium of rats during the estrus cycle and under the influence of administered estrogens. The alkaline phosphatase activity of the oral epithelium was found to increase during the estrus cycle, with its maximum during estrus. While the authors found that the gingival epithelium has only a negligible phosphatase activity during all stages of the estrus cycle and even in the presence of administered estrogens, they did feel that the distribution of the zones of activity in the oral epithelium suggest a relationship to the process of keratin formation. It is unfortunate that the authors did not examine smears from the oral cavity at the same time they examined the daily vaginal smears during the estrus cycle. The findings, however, offer a possible explanation for some of the oral epithelial changes observed during the estral cycle in women, and associated with disturbances of the estrus under the influence of administered estrogens.

Seltzer and Bender (p. 825) tested a number of antibiotics against enterococci. These organisms are resistant to penicillin and streptomycin and are found in 6 to 8 per cent of all infected root canals. Chloromycetin was effective against enterococci in quantities practical for root canal therapy. The authors did not find a useful antifungicide, although 19 per cent of infected root canals include fungi. They suggested that a mixture of penicillin, streptomycin and chloroamphenical (Chloromycetin) be used in endodontic therapy and, when discovered, an effective fungicide be added.

Shafer (p. 831) conducting work similar to that reported by Gorlin (*J. D. Res.* **29**: 208, 1950) was unable to demonstrate scurvy in rats, hamsters, and guinea pigs fed glucoascorbic acid. These histologic studies both refute the laboratory observation of a "scurvylike" condition in animals fed glucoascorbic acid and demonstrate the importance of thorough study of laboratory material.

Medak, Schour, and Klauber (p. 839) directed single large doses of irradiation at the base of the upper incisors of young albino rats. With 2000 to 2500 $\mu$, some showed increase in eruption rate and others showed decelaration but at 3000 to 4000 $\mu$, eruption decreased and finally ceased. The effect was directly on the odontogenic apparatus. This effect helps to explain the results observed after therapeutic irradiation of children's jaws as in the case described by Brown (*J. A. D. A.* **38**: 754, 1949).

The American College of Dentists has set up two funds which will be helpful to those engaged in dental research. Grants will be made from the William J. Gies Re-

search Travel Fund to investigators who wish to visit the laboratory of a contemporary worker for the purpose of obtaining specific information relating to his own problem. Assistance will be given to the extent of the cost of first class transportation and a modest per diem. The second fund was established to cope with emergencies which sometimes arise in research laboratories. It is planned to provide speedy financial assistance where accidents to animal colonies or equipment threaten the termination of studies already in progress. Applications for grants from either of the funds should be made to Dr. Phillip Jay, Chairman of the Committee on Research of the American College of Dentists, School of Dentistry, University of Michigan, Ann Arbor, Mich.

An unusual publication which has been prepared for and issued by the Associate Committee on Dental Research, National Research Council, Ottawa, Canada, is *Bibliography on Caries Research*. This reference work of 577 pages is a thorough review of the literature on caries from 1890 to 1949 and is arranged as a useful series of abstracts with a bibliography of 2,300 publications. The volume will be useful to students and investigators in the field of dental caries. The edition is limited to 300 copies available from the National Research Council, Ottawa, Canada, at $10.00 each.

H. B. G. R.

# THE PROBLEM OF GROUP SPECIFICITY IN ALLERGY TO LOCAL ANESTHETICS

P. ADLER, L. KESZTYÜS, AND N. SIMON

*From the Institute of Physiology and of Experimental Pathology; the Stomatological Clinic; and the Dermato-Syphilological Clinic of the University, University of Debrecen, Debrecen, Hungary*

THE problem of allergy to local anesthetics in general and to procaine in particular is of a vital importance to workers in those who employ local anesthesias. In most reports on procaine dermatitis, the patients are dentists; procaine dermatitis is regarded as a common occupational disease of dentists.[1]

Following early publications upon this topic, contradictory views of the exciting cause were expressed in the literature. First the condition was described as being caused by procaine but subsequently it was suggested that the dermatitis is caused by other irritants. It became a well-established fact that these rare cutaneous reactions are due to an allergic hypersensitivity to one of the drugs used in local anesthesia, mostly commonly to procaine. The passive transmittibility of the condition from one person to a nonallergic one is cogent evidence of the allergic nature of the phenomenon.[2] One problem remained, however, unsolved: What drug can be used with safety by the dentist who is allergic to one of the local anesthetics? This question is of the utmost practical importance, since a procaine dermatitis can markedly handicap the dentist in his work. The literature is confusing. Goodman[3] reported a strong group specificity, allergy being present to derivatives of para-aminobenzoic acid (PABA), but not to ortho- and meta-aminobenzoic acid derivatives. More recently, the allergy has been restricted by Rothman, Orland, and Flesch[4] to derivatives of PABA with a secondary or tertiary amine group on the side chain. Simon and Adler,[5] after testing a very few drugs in 3 patients, confirmed the findings of Goodman[3]: All the patients were allergic to procaine and tetracaine and 2 who were tested with PABA were allergic to it. Laden and Rubin[6] found eczematous hypersensitivity to the simple alkyl esters of PABA and no allergy to procaine and its homologues in a 15-year-old boy. Strauss's[7] patient exhibited marked allergy to tetracaine, a slight hypersensitivity to Apothesine Hydrochloride but no allergy to procaine. Laden and Wallace[1] using patch tests on 8 dentists found the allergy mainly restricted to the procaine group, extended in a few instances only to simple alkyl esters of PABA.

In discussing a case of allergy to procaine by correspondence with Dr. Lon W. Morrey (September, 1948-January, 1949), the senior author became aware of the inadequacy of examining this problem solely from the standpoint of clinical experience in man. In spite of increasing frequency of the use of large procaine dosages (in medical therapy) and of the accumulated knowledge of the phenomenon, the scope of clinical experience is limited. First of all, any single investigator rarely sees many cases; second, allergy in man is displaying

TABLE I

LIST OF DRUGS EXAMINED AND OF THEIR EFFECTS IN PRECIPITATION INHIBITION TEST WITH PROCAINE-AZOPROTEIN IMMUNE SERUM OF RABBITS

| DRUG | CHEMICAL FORMULA OF THE BASE | DEGREE OF INHIBITION |
|---|---|---|
| 1. Simple esters of para-aminobenzoic acid: | | |
| A. Ethyl-ester (anesthesin) | $NH_2—C_6H_4—COO·C_2H_5$ | Partial |
| B. Normopropyl-ester | $NH_2—C_6H_4—COO·C_3H_7$ | Partial |
| C. Normobutyl-ester | $NH_2—C_6H_4—COO·C_4H_9$ | Partial |
| 2. Esters of para-aminobenzoic acid with an amine group in the side chain (procaine and its higher homologues): | | |
| A. Procaine hydrochloride | $NH_2—C_6H_4—COO·CH_2—CH_2—N=(C_2H_5)_2$ | Total |
| B. Larocaine hydrochloride | $NH_2—C_6H_4—COO·CH_2—C(CH_3)_2—CH_2—N=(C_2H_5)_2$ | Total |
| C. Butyn (butacaine sulfate) | $NH_2—C_6H_4—COO·CH_2—CH_2—CH_2—N=(C_4H_9)_2$ | Partial |
| D. Monocaine (butethamine) | $NH_2—C_6H_4—COO·CH_2—CH_2—NH—CH_2—CH=(CH_3)_2$ | Partial |
| E. Tutocaine hydrochloride | $NH_2—C_6H_4—COO·CH(CH_3)—CH(CH_3)—CH_2—N=(CH_3)_2$ | Partial |
| F. Panthesine | $NH_2—C_6H_4—COO·CH_2—CH=N—CH_2—CH=(CH_3)_2$ with $(C_2H_5)_2$ | Partial |
| G. Pontocaine hydrochloride (tetracaine) | $H_9C_4—NH—C_6H_4—COO·CH_2—CH_2—N=(CH_3)_2$ | None |
| 3. Derivatives of benzoic acid and of oxybenzoic acid: | | |
| A. Cocaine | benzoyl-ecgonin | None |
| B. Stovaine | $C_6H_5—COO·C(=(CH_3)_2)(C_2H_5)—CH_2—N=(CH_3)_2$ | None |
| C. Alypin hydrochloride | $C_6H_5—COO·C(CH_2—N=(CH_3)_2)_2$ | None |
| D. Metycaine (piperocaine) | $C_6H_5—COO—CH_2—CH_2—CH_2—NC_5H_9—CH_3$ | None |
| E. Intracaine | $C_2H_5—O—C_6H_4—COO·CH_2—CH_2—N=(C_2H_5)_2$ | None |
| F. Orthoform | $HO—C_6H_3(NH_2)—COO·CH_3$ | None |
| 4. Derivatives of other compounds than benzoic acid: | | |
| A. Derivatives of phenol: | | |
| 1. Acoin | $(CH_2—O—C_6H_4—NH)_2=C=N—C_6H_4—O—C_2H_5$ | None |
| 2. Phenacaine hydrochloride (holocaine) | $C_2H_5—O—C_6H_4—N=C(CH_3)—NH—C_6H_4—O—C_2H_5$ | None |
| B. Derivative of cinnamic acid: | | |
| 1. Apothesine hydrochloride | $C_6H_5—CH=CH—COO·CH_2—CH_2—CH_2—N=(C_2H_5)_2$ | None |
| C. Derivative of isoquinoline: | | |
| 1. Nupercaine hydrochloride (dibucaine) | $C_4H_9—O—C_9H_6N—O—NH—CH_2—CH_2—N=(C_2H_5)_2$ | None |

TABLE I—CONT'D

| DRUG | CHEMICAL FORMULA OF THE BASE | DEGREE OF INHIBITION |
|---|---|---|
| D. Derivative of xylidine: | | |
| 1. Xylocaine (lidocaine) | $(CH_3)_2=C_6H_2—NH—CO\cdot CH_2—N=(C_2H_5)_2$ | None |
| E. Diothane hydrochloride (diperodon) | $H_{10}C_5N—CH_2—CH—COO—NH—C_6H_5$ <br> $\quad\quad\quad

tetracaine. This finding contradicts the clinical findings of Simon and Adler[5] and of Laden and Wallace[1] (that cocaine and local anesthetics derived from other nucleus than PABA fail to inhibit precipitation was in best agreement with clinical experience). In this respect, the behavior of Intracine and procaine deserves special attention. These drugs have as single difference an ethoxy group in Intracaine and an amino group in procaine in "para" position to the carboxyl group on the benzene ring. Procaine displayed maximal inhibition in all sera, while Intracaine causes no inhibition. Furthermore, while the simple esters of PABA displayed marked inhibition, orthoform failed to inhibit precipitation, its carboxyl and amino groups not being in *p* position.

It is questionable whether or not cutaneous hypersensitivity to local anesthetics is connected in any way with a precipitation reaction. On the other hand, it can be assumed that inhibition of precipitation has nothing to do with clinical allergic phenomena. Inhibition of precipitation as well as cutaneous allergy are manifestations of reactions to the drugs. Thus, to a certain degree, we are justified in amplifying the results of these experiments, at least so far as to exclude all those local anesthetics from use by a dentist who is allergic to any one drug of the PABA series that gave an inhibition of precipitation. This conclusion is in agrement with the final statement of Laden and Wallace.[1]

We are unable to explain satisfactorily the difference between the reaction of tetracaine in precipitation inhibition tests on the serum of rabbits immunized by procaine-azoprotein, and behavior in our clinical patients.[5] Similarly, we cannot explain why PABA itself caused cutaneous allergic reactions in our patients and failed to inhibit precipitation. The negativity of the precipitation inhibition tests against procaine-azoprotein-antiserum with tetracaine and PABA may be due to the fact that our rabbits were not sensitized to the same degree as are clinical patients. A further possible explanation of this discrepancy may be the different sensitivity of the skin as compared with in vitro tests of the serum. However, none of these conjectures is proved. Based on our experiments, we are limited to the statement that precipitation is inhibited by all esterified derivatives of PABA in which the aromatic amino group is free of side chain.

That procaine-azoprotein has a definite connection to cutaneous allergy to procaine, has been demonstrated by Adler and Simon.[12] A patient allergic to procaine and tetracaine proved to be allergic to procaine-azoprotein as well. By injecting procaine mixed with the serum of rabbits immunized with procaine-azoprotein, in proper quantative proportions, no allergic cutaneous reaction was excited. By injecting the antiserum of rabbits into the site of a previous intracutaneous procaine injection (that caused an allergic eruption) attenuation was observed.

## SUMMARY

Precipitation inhibition tests on a procaine-azoprotein antiserum of rabbits with different local anesthetic drugs gave evidence of a strong group specificity. Inhibition was restricted to those esterified derivatives of PABA in which the

aromatic amino group has no side chain. To dentists allergic to one anesthetic of the procaine series, the use of local anesthetics with a different chemical structure and not derivatives of para-aminobenzoic acid is recommended.

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# A BACTERIOLOGIC SURVEY OF GINGIVAL SCRAPINGS FROM PERIODONTAL INFECTIONS BY DIRECT EXAMINATION, GUINEA PIG INOCULATION, AND ANAEROBIC CULTIVATION

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THERE is much indirect evidence that a characteristic mixed anaerobic infection plays an indispensable role in the common pathologic processes involving one or more of the supporting tissues of the teeth, e.g., marginal gingivitis, ulcerative gingivitis (Vincent's infection), and periodontitis. Exudates or scrapings from the lesions of any of these processes, when inoculated parenterally into guinea pigs or other animals, frequently yield transmissible infections that are clearly related bacteriologically to the original disease.[16, 37, 43, 51] This experimental infection has similar characteristics even though the inoculum be derived from lesions in man that differ widely in their localization in the periodontal tissues and in their chronicity and severity.[7] The beneficial results that have followed penicillin therapy in these diseases,[31] moreover, point to the significance of infection as an inciting cause of the symptoms. On the other hand, the marked tendency of these diseases to recur following such therapy indicates the existence of deeper causative factors that appear to differ with each clinical entity. It has been suggested[32] that infection, similar in kind for the different diseases, is one factor among several in their complex etiology, the others being distinct and accounting for the clinical differences.

The infection itself, however, remains inadequately defined. Since exudates obtained after repeated passage through guinea pigs are preponderantly if not exclusively anaerobic,[37] the data reported in many studies of the periodontal tissues based on aerobic cultures[1] seem of little value. Although many previous workers have isolated and described one or a few of the anaerobic species found in these diseases, e.g., spirochetes,[22, 26, 38, 41, 43] fusiform bacilli,[14, 42, 45, 48, 50] anaerobic streptococci,[13, 20, 23, 24] *Bacteroides*,[2, 17, 18, 44] vibrios,[21, 25, 27, 49] actinomycetes,[30, 53, 54] *Leptotrichia*,[46, 52] *Veillonella*,[8, 9, 19] and other forms[28, 47] the writers know of no adequate attempt to explore the whole anaerobic flora of periodontal disease by cultural means. Veillon and Zuber[50] seem to have been the first to apply adequate anaerobic methods for the isolation of bacteria from the mouth and from pathologic processes associated with other mucous membranes. Their emphasis, however, like that of many subsequent workers, was on the individual types isolated rather than on the anaerobic flora as a whole. Brailovsky-Lounkevitch[5] studied the mouth flora in "pyorrhea" by anaerobic methods, and noted the constant appearance of forms characteristics of the normal adult mouth. More recently, Hemmens and Harrison[11] studied that distribution of bacterial types in gingival scrapings from 46 cases of suppurative

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periodontitis and from 27 normal cases, using streaked sheep blood agar plates incubated anaerobically. Their data on spirochetes were based on microscopic examination only. These workers recovered Fusobacterium (recorded as *Fusiformis nucleatus* Hine and Berry[14] and "other fusiform bacilli," apparently on morphological criteria), *Veillonella (Micrococcus) gazogenes, Bacteroides melaninogenicum* and other "bacteroids," gram-positive cocci, and Leptotrichia. The incidence was found to be greater in periodontal disease than in normal mouths only of spirochetes, "other fusiform bacilli," and *Bacteroides melaninogenicum.*

The present study is the initial phase of a broader investigation of the potentially pathogenic anaerobes of the mouth, other phases of which are reported separately.[33, 34, 35, 39, 40] Its primary purpose was the isolation of the widest possible range of anaerobes from the gingival and periodontal area for studies of individual and combined pathogenicity. The principal data, on the results obtained in anaerobic cultures, are based on 35 cases of gingival or periodontal disease studied during the 3-year period, 1946-1949. To these have been added earlier findings from this laboratory, hitherto unpublished, dealing with the results of direct examination and of guinea pig inoculation of such gingival scrapings. Thus assembled the data permit certain comparisons of different gingival and periodontal diseases, some of which have not to the authors' knowledge been possible heretofore.

## METHODS

Gingival scrapings were taken with scalers from unselected cases of gingival and periodontal disease in the Diagnostic Clinic of the School of Dental and Oral Surgery, Columbia University.* Clinical diagnosis and other data pertaining to each subject were recorded by members of the Dental School staff on forms provided by this laboratory. The scrapings were suspended as they were taken from each mouth in 1 ml. of extract broth, a sufficient amount of material being taken to make a visibly turbid suspension. For a group of normal subjects, 0.5 ml. of extract broth was used similarly. Within 2 hours after being taken, the suspensions were examined microscopically by dark-field illumination, cultured by the methods indicated below, and, in some instances, inoculated into guinea pigs.

*Dark-field Examination.*—This examination was facilitated by the use of a record form bearing categories similar to those listed in Table I. The different categories were identified morphologically in line with the following criteria:

*Treponema microdentium.* Tightly wound spirochetes similar to *T. pallidum* in appearance, but usually with shallower coils; single contoured; motility when present actively rotatory, frequently with marked translation; movements of flexion stiff, jerky.[29]

*Intermediate spirochetes.* A miscellaneous group including forms intermediate in wave length between *T.microdentium* and *T.vincenti*, and forms showing spiral-on-spiral winding ("double spirals"); single contoured; motility various, usually resembling that of *T.microdentium.*

*Treponema* (or *Borrelia*) *vincenti.* Loosely wound single-contoured spirochetes, generally somewhat longer than the short forms of *T.microdentium;* motility when present

*Dr. L. R. Stone, Dr. E. V. Zegarelli, and other members of the staff of this clinic cooperated to provide this material.

TABLE I

MICROORGANISMS SEEN BY DARK-FIELD EXAMINATION IN GINGIVAL SCRAPINGS FROM 136 CASES OF GINGIVAL OR PERIODONTAL INFECTION AND FROM 10 NORMAL MOUTHS

| | CLINICAL DIAGNOSIS | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NORMAL GINGIVAE | | VINCENT'S GINGIVITIS | | PERIODONTOCLASIA | | MARGINAL GINGIVITIS | | PERICORONITIS | | HERPETIC GINGIVOSTOMATITIS | |
| | NO. | % | NO. | % | NO. | % | NO. | % | NO. | % | NO. | % |
| Number of cases | 10 | | 52 | | 39 | | 22 | | 10 | | 13 | |
| *Treponema microdentium*, many | 0 | 0 | 35 | 67 | 20 | 51 | 9 | 41 | 7 | 70 | 8 | 62 |
| *T. microdentium*, few | 3 | 30 | 17 | 33 | 18 | 46 | 13 | 59 | 3 | 30 | 5 | 58 |
| Intermediate spirochetes, many | 0 | 0 | 26 | 50 | 16 | 41 | 5 | 23 | 3 | 30 | 4 | 31 |
| Intermediate spirochetes, few | 5 | 50 | 25 | 48 | 22 | 56 | 15 | 68 | 7 | 70 | 6 | 46 |
| *T. vincenti*, many | 0 | 0 | 36 | 69 | 24 | 62 | 9 | 41 | 5 | 50 | 4 | 41 |
| *T. vincenti*, few | 5 | 50 | 15 | 29 | 15 | 38 | 12 | 55 | 5 | 50 | 9 | 69 |
| *T. buccale*, many | 0 | 0 | 38 | 73 | 18 | 46 | 6 | 27 | 3 | 30 | 2 | 15 |
| *T. buccale*, few | 4 | 40 | 13 | 25 | 20 | 51 | 16 | 73 | 5 | 50 | 6 | 46 |
| Leptospiras | 0 | 0 | 4 | 8 | 11 | 28 | 6 | 27 | 0 | 0 | 0 | 0 |
| Vibrios, many | 0 | 0 | 41 | 79 | 28 | 72 | 15 | 68 | 6 | 60 | 6 | 46 |
| Vibrios, few | 7 | 70 | 11 | 21 | 11 | 28 | 6 | 27 | 4 | 40 | 7 | 54 |
| Fusiforms, many | 0 | 0 | 35 | 67 | 23 | 59 | 13 | 59 | 3 | 30 | 9 | 69 |
| Fusiforms, few | 10 | 100 | 17 | 33 | 16 | 41 | 9 | 41 | 6 | 60 | 4 | 31 |
| Large "fusiforms" | 0 | 0 | 37 | 71 | 29 | 74 | 12 | 55 | 7 | 70 | 8 | 62 |
| Motile "fusiforms" | 1 | 10 | 3 | 6 | 6 | 15 | 3 | 14 | 3 | 30 | 0 | 0 |
| Spirilla | 3 | 30 | 29 | 56 | 29 | 74 | 8 | 36 | 6 | 60 | 10 | 77 |
| Motile bacilli | 8 | 80 | 28 | 54 | 28 | 72 | 13 | 59 | 7 | 70 | 9 | 69 |
| Nonmotile bacilli | 8 | 80 | 45 | 87 | 34 | 87 | 16 | 73 | 6 | 60 | 9 | 69 |
| Unbranched filaments | 8 | 80 | 25 | 48 | 25 | 64 | 14 | 64 | 6 | 60 | 6 | 46 |
| "Twigs" or branched filaments | 7 | 70 | 1 | 2 | 12 | 31 | 6 | 27 | 3 | 30 | 3 | 23 |
| Cocci, many | 2 | 20 | 23 | 44 | 20 | 51 | 11 | 50 | 4 | 40 | 10 | 77 |
| Cocci, few | 8 | 80 | 29 | 56 | 19 | 49 | 11 | 50 | 6 | 60 | 3 | 23 |
| "Vibriothrix" forms | 0 | 0 | 0 | 0 | 3 | 8 | 0 | 0 | 0 | 0 | 0 | 0 |
| Trichomonads | 0 | 0 | 1 | 2 | 2 | 5 | 1 | 4 | 0 | 0 | 0 | 0 |
| Amoebae | 0 | 0 | 1 | 2 | 1 | 3 | 1 | 4 | 0 | 0 | 0 | 0 |
| Leucocytes, many | 0 | 0 | 9 | 17 | 14 | 36 | 3 | 14 | 0 | 0 | 0 | 0 |

actively rotatory with alternate stretching and relaxation of coils; translation active; flexion not prominent.[29]

*Treponema* (or *Borrelia*) *buccale.* Similar to *T. vincenti* in morphology and motility, but double contoured.

*Leptospiras.* Forms morphologically typical of this genus; more tightly wound than *T.microdentium*, with one or both ends hooked; single contoured.

*Vibrios.* Small (less than 1 $\mu$ × less than 6 $\mu$) curved, crescentic or comma-shaped rods, sometimes showing one polar or subpolar flagellum, with typical vibratory or tumbling motility.

*Fusiforms.* Straight rods with delicately tapered ends, usually 0.5 $\mu$ wide or less in the widest portion, and 10 $\mu$ or less long; with or without granules; nonmotile; often in chain or "tandem" pairs.

*Large fusiforms.* Similar to the above but up to 1 $\mu$ wide and 12-15 $\mu$ long; generally more bluntly tapered than the small fusiforms; nonmotile.

*Motile "fusiforms."* Motile forms morphologically similar to the smaller fusiforms motility by slow progression.

*Spirilla.* At least three varieties: (1) like the vibrios in form and motility but larger (1-1.2 $\mu$ × 8-10 $\mu$); flagella, when present, subpolar on the concave side, sometimes peritrichous; (2) straight or slightly curved motile cigar-shaped forms, 1 $\mu$ or more × 10 $\mu$ or more, often barred or granular; flagella, when present, peritrichous; and (3) spiral forms with rounded ends, about 1 $\mu$ wide × 8 $\mu$ or more with 2 to 5 shallow coils, actively motile. Flagella have not been seen on the last variety.

*Motile bacilli.* Various forms not included in the above.

*"Twigs" or branched filaments.* Include forms probably belonging in both aerobic and anaerobic genera of actinomycetes.

*Cocci.* Single cocci, diplococci, streptococci, and clumps of these or of other coccal types, not easily distinguishable in dark-field preparations.

*"Vibriothrix"[47] or "falciform"[4] types.* Filaments or stalks surrounded by radially arranged fusiform elements; "test-tube brush" forms.

*Guinea Pig Inoculation.*—Inoculation was done by the subcutaneous route in the groin area, using the method previously described.[37]

*Cultural Methods.*—Inoculum for cultures was taken either directly from suspensions of gingival scrapings or from exudate obtained by aspiration of a guinea pig lesion after several passages through guinea pigs. In some instances, to be described in detail in another report,[34] inoculum for plating was taken after passage of mixed cultures anaerobically on sheep blood agar or in the "AFK cup plates" mentioned below.

The three media used routinely for isolation of aerobes were (1) sheep blood agar—beef heart infusion agar containing 10 per cent of defibrinated sheep blood; (2) GV agar—a medium containing veal infusion agar (Difco) to which was added aseptically ascitic fluid, 30 per cent, glucose, 0.2 per cent, neutralized cysteine HCl, 0.1 per cent, and gentian violet, 0.003 per cent (titrated to pH 7.3 to 7.5); and (3) AFK agar—the Proske-Sayers[26] "hormone" agar containing ascitic fluid, 30 per cent, neutralized cysteine HCl, 0.1 per cent, adjusted to pH 7.3 to 7.5 and poured over a few small pieces of fresh sterile guinea pig kidney. Blood agar was used in standard Petri plates, GV agar in small Petri plates (13 mm. deep × 50 mm. inside diameter), and AFK agar in special deep Petri dishes,[40] 30 mm. deep × 25 mm. inside diameter, filled to a depth of about 25 mm.

Other media used intermittently or for comparative tests included brain-heart agar (Difco) alone or with 10 per cent ascitic fluid; the GV medium as given but without gentian violet; and the AFK medium without kidney, all in standard Petri plates. The last two media have also been used in the small deep dishes for isolation of spirochetes, as noted below. For isolation of gram-negative organisms other than Fusobacterium, particularly vibrios, attempts have also been made to use several types of enrichment media employing gentian violet (0.001 per cent), brilliant green (1:5,000 to 1:10,000); sodium azide (0.01 per cent), penicillin G (0.05 to 10 units per ml.); aspergillic acid (1:1,000 to 1:280,000) or bacitracin (5 to 40 units per ml.). Since none of these alternative media or methods proved conspicuously selective or advantageous, the data are not reported in further detail. All organisms obtained in these studies could be grown on or in one or more of the three media mentioned in the preceding paragraph. All these forms, in fact, except the spirochetes and certain of the vibrios, grew anaerobically as surface colonies on sheep blood agar plates. For isolation of spirochetes the AFK medium has been found best of those tried; this medium has also been used for isolation of vibrios and Leptotrichia, and as an aid in the isolation of other forms. The GV medium is markedly selective for Fusobacterium.

Sheep blood agar and other nonselective media were streaked serially, using two or three plates streaked successively with a single small loopful of inoculum. The GV medium and other selective media in small plates were streaked after inoculation with one drop of undiluted gingival scrapings or guinea pig fuso-spirochetal exudate. The AFK plates were inoculated, as previously described,[38] in a well or cup in the center of the upper surface, the well having been prepared with a sterile thin-walled glass tube of 2 mm. diameter attached to a small syringe. The inoculum was either dropped into the well with a capillary pipette or stabbed into its floor with a straight needle, care being taken by either method to avoid contaminating the upper surface of the medium. This method for separation of spirochetes from bacteria, developed in this laboratory, has been used successfully with modifications by Hampp.[10]

All media were tested for sterility by incubating at 37° C. under aerobic conditions for 24 hours, then under anaerobic conditions for 48 hours or more, and were stored in anaerobic jars until needed. Inoculated plates were incubated at 37° C. for 2 to 9 days in Brewer or equivalent anaerobic jars, anaerobiosis being obtained with hydrogen, with 5 per cent of $CO_2$ added. Successful anaerobiosis was determined by reduction of methylene blue, prepared as a dilute solution of the dye in 2 per cent glucose-phosphate buffer, pH 8.3.

Organisms isolated as surface colonies were either fished directly to fluid media or purified by restreaking on the same agar medium. Of several fluid media tested, most consistent results were obtained with Difco thioglycollate broth (No. B36) incubated anaerobically. Enrichment of this medium with 10 per cent of ascitic fluid and 0.1 per cent of cysteine HCl has been found to enhance the growth of some forms, but did not seem essential. Growth in the Difco B36 medium prepared without agar was generally less abundant than in the complete medium, but this modification was useful when centrifugation was to be used to concentrate the culture.

Spirochetes, certain vibrios, and some strains of Leptotrichia were isolated from AFK cup plates by aspirating, with a capillary pipette through the sterile upper surface of the medium, portions of outgrowths into the agar, the outgrowths being well separated from the central mixed growth. This inoculum was transferred with the pipette to AFK medium in Noguchi tubes (10 × 200 mm.), being ejected into the medium gently without introducing air. Both aspiration and ejection were accomplished with the aid of a dry, clean 5 ml. Luer syringe attached to the capillary pipette with a short length of rubber tubing. Vibrios and Leptotrichia isolated by this means were subsequently transferred to thioglycollate broth.

Purity of spirochete cultures and of certain vibrios that failed to grow as surface colonies could be determined only by lack of growth on subculture by both aerobic and anaerobic plating technics. Such cultures could not be shown to be bacteriologically pure.[40] With other organisms, purity was checked by plating on sheep blood agar both aerobically and anaerobically. Except for Leptotrichia and actinomycetes, some strains of which were capable of limited growth under aerobic conditions, and for certain streptococci isolated from guinea pig exudates, the organisms cultured in this work all failed to grow when plated aerobically.

Identification of organisms was undertaken only broadly on the basis chiefly of morphologic criteria, including colony form, cell morphology and motility, and gram-staining reaction. Biochemical tests were performed on selected cultures.[34] To meet the primary purpose of this investigation, the isolation of oral anaerobes for subsequent study, the cultures, thus roughly identified, were stored in the earliest available pure subculture by freezing in $CO_2$ ice. For this purpose, broth cultures were filled directly into 1 ml. ampules and sealed in a flame; agar cultures (e.g., spirochetes) were treated similarly after being emulsified in small volumes of extract broth in glass tissue grinders. The stability of these frozen cultures is considered elsewhere.[39]

## RESULTS

*Dark-field Examination.*—The findings in dark-field examination of gingival scrapings from 146 mouths are assembled in Table I, classified on the basis of clinical diagnosis and of the morphologic types defined above. The pathologic samples represent a compilation of all complete records for gingival or periodontal disease made in this laboratory since 1940, except for small numbers of cases with diagnoses other than those listed. The 10 normal cases were examined on a single day as a comparative group. These 10 subjects were selected by Dr. E. V. Zegarelli from among the personnel and students of the Diagnostic Clinic of the School of Dental and Oral Surgery. All had clinically normal gingival tissues, without visible hyperemia, inflammation, pocket formation, or resorption.

Since these findings depend upon observation of a variety of morphologic types in a complex mixture, they tend to err on the side of omission. A given type may have been overlooked or missed even though present. Such error was minimized but not abolished by careful observation in which each type was looked for and recorded on a form on which the types were listed. Distinctive morphology and motility, or both, facilitated observation; and the findings are unavoidably weighted to some degree by this fact. Such forms as amoebae, moreover, were seen seldom and were not routinely looked for, so that their indicated prevalence is doubtless too low.

The absence in the smaller series (normals, pericoronitis, and herpetic gingivostomatitis) of such forms as leptospiras and protozoa may accordingly not be significant. Similarly insignificant may be the absence of large "fusiforms" in the normal group. With these exceptions, all the types found under pathological conditions were found as well in 1 or more of the normal cases. Spirochetes of one or more types appeared in 7 out of 10 of these. The normal picture would thus seem to be distinctive not in that it lacks forms seen in disease but rather in the relative scarcity of many types. Spirochetes were never numerous in the normal samples; indeed the arbitrary distinction used here between "many" and "few" does not indicate the marked quantitative difference in prevalence of these forms under normal as compared with pathologic conditions. In the latter, even when these organisms were recorded as "few," they were invariably far more numerous than in any of the normal samples. Similar differences apply to most of the other types. The whole flora of the normal gingival area is scanty as compared with the pathologic flora.

Comparison of the data for the three most common diseases listed, Vincent's gingivitis, periodontoclasia, and marginal gingivitis, shows a tendency toward reduction of all the treponemas in the order of these diseases as given. All four varieties of these spirochetes appeared in nearly all instances in these groups, the exceptions being again probably attributable to oversight rather than to absence. The prevalence of vibrios and true fusiforms was similar in the three diseases. On the other hand, certain forms were notably more prominent in periodontoclasia and, in less degree, in marginal gingivitis than in Vincent's gingivitis: particularly leptospiras, actinomycete-like forms, and "vibriothrix" forms. Together with the slightly higher prevalence in periodontoclasia of atypical "fusiforms," spirilla, motile bacilli, unbranched filaments, and undifferentiated cocci, these findings point to a tendency in this disease toward a more mixed and less typically "fusospirochetal" flora. This difference, which was mentioned in an earlier report from this laboratory,[7] seems to be a statistical one, and is not in the writers' experience sufficiently marked to have diagnostic value. Although the types just listed are less likely to be prominent in Vincent's infection than in periodontoclasia, the data indicate that most if not all of them may be found in both; and many individual preparations made from the two diseases have seemed quite undistinctive.

TABLE II

FREQUENCY OF TYPICAL FUSOSPIROCHETAL RESPONSES IN GUINEA PIGS INOCULATED SUBCUTANEOUSLY WITH GINGIVAL SCRAPINGS FROM DIFFERENT GINGIVAL AND PERIODONTAL DISEASES

| CLINICAL DIAGNOSIS | NEW DATA | | POOLED WITH EARLIER DATA[7] | |
|---|---|---|---|---|
| | INOCULATED/ INFECTED NUMBER | PER CENT INFECTED | INOCULATED/ INFECTED NUMBER | PER CENT INFECTED |
| Vincent's gingivitis | 11/12* | 92 | 28/41 | 68 |
| Periodontoclasia | 7/9 | 78 | 23/34 | 68 |
| Marginal gingivitis | 6/7 | 86 | 11/13 | 85 |
| Herpetic gingivostomatitis | 9/12 | 75 | 9/12 | 75 |
| Pericoronitis | | | 2/4 | 50 |
| Totals | 33/40 | 82 | 73/104 | 70 |

*11/12, etc., = 11 typical fusospirochetal responses out of 12 inoculations.

The occurrence of "vibriothrix" forms[4,7] only in periodontoclasia may support the views of Box[4] that these organisms have distinctive significance in this disease. Their low prevalence in our experience is unlikely to be due to oversight, because of their striking morphology. It seems possible but unlikely that they may be broken up and hence made unrecognizable in the wet preparations used for dark-field examination. These forms have not been seen either in cultures or in the exudates from fusospirochetal lesions in experimental animals.

The data for pericoronitis and for herpetic gingivostomatitis, in view of the small size of both groups, may be said to indicate only that these diseases show a flora characteristic of gingival and periodontal disease generally.

*Guinea Pig Inoculation.*—Table II presents new data on the occurrence of typical fusospirochetal responses in guinea pigs inoculated subcutaneously with gingival scrapings from four of the clinical types of gingival and periodontal

disease. Earlier data from this laboratory[7] indicated that such responses could be elicited in from 59 to 83 per cent of trials with scrapings from the first three diseases listed, and in 50 per cent of trials with pericoronitis. The present findings confirm the earlier ones for marginal gingivitis, periodontoclasia, and Vincent's gingivitis, and indicate that scrapings from herpetic gingivostomatitis have comparable infectivity for guinea pigs. The combined data in the last two columns of Table II suggest that scrapings from any one of these processes are not significantly more or less infective than those from any other.

TABLE III

MICROORGANISMS ISOLATED FROM 35 CASES OF GINGIVAL OR PERIODONTAL INFECTIONS AND FROM 4 CASES OF SUSPECTED CERVICOFACIAL ACTINOMYCOSIS

| | CLINICAL DIAGNOSIS | | | | | | | ISOLATED FROM | |
|---|---|---|---|---|---|---|---|---|---|
| | VINCENT'S GINGIVITIS | PERIODONTOCLASIA | MARGINAL GINGIVITIS | PERICORONITIS | MISCELLANEOUS* | SUSPECTED ACTINOMYCOSIS† | TOTALS | GINGIVAL SCRAPINGS | GUINEA PIG EXUDATES |
| Number of cases | 6 | 12 | 10 | 3 | 4 | 4 | 39 | 39 | 4 |
| Spirochetes, tightly wound | 3/2‡ | 3/2 | 1 | 1 | 1 | | 9/7 | 3/3 | 6/4 |
| Spirichetes, intermediate or variable | 9/4 | 3/3 | 3/2 | | 1 | | 16/10 | 10/8 | 6/3 |
| Fusobacterium | 10/4 | 11/8 | 9/6 | 1 | 8/4 | 1 | 40/24 | 32/22 | 8/2 |
| Anaerobic vibrios | 5/1 | 2/2 | 3/2 | | | | 10/5 | 5/4 | 5/1 |
| Bacteroides | 10/3 | 4/3 | 7/3 | 3/1 | | 4/2 | 28/12 | 17/10 | 11/2 |
| Leptotrichia | 3/2 | 1 | 2/2 | 1 | | | 7/6 | 7/6 | |
| Actinomyces | 1 | 1 | 1 | | | 3/3 | 6/6 | 6/6 | |
| Anaerobic streptococci | 3/1 | 4/3 | 8/3 | | | 2/1 | 17/8 | 9/5 | 8/4 |
| Veillonella | 2/2 | 2/2 | 2/1 | | 2/2 | | 8/7 | 8/7 | |
| Anaerobic gram-positive bacilli | 11/4 | 7/6 | 15/7 | 3/2 | 3/2 | 1 | 40/22 | 29/20 | 11/2 |
| Anaerobic gram-negative threads | 1 | 2/1 | | 2/1 | | | 5/3 | 5/3 | |

*Included 2 cases of parietal dental abscess, 1 of herpetic gingivostomatitis, and 1 of osteomyelitis following tooth extractions.

†The inoculum for cultures in these cases consisted of pus aspirated or drained through the skin of the cheek or mandibular area under aseptic precautions (cases of Dr. J. Schroff).

‡3/2, etc., = 3 isolations from 2 cases.

*Cultures.*—Data on the distribution of pure or separated cultures of anaerobes are assembled in Table III on the bases of (1) 39 samples of gingival scrapings classified clinically, and (2) the same samples classified as to whether the isolations were made from the original scrapings directly or from exudates obtained from lesions in guinea pigs inoculated with four of these. The types of organisms isolated, as listed in the table, may be defined as follows:

*Spirochetes.* These forms are grouped in two categories, "tightly wound" (presumably *T.microdentium*) and "intermediate or loosely wound" (possibly including several species). Morphologic criteria alone do not permit further subdivision with confidence; indeed, even this division is offered only tentatively pending additional study of these microorganisms. Studies with these spirochetes are presented elsewhere.[40]

*Fusobacterium.* These organisms conform morphologically and, so far as they have been studied, in other respects as well, with the criteria defined by Bøe.[3] They are small or medium-sized gram-negative fusiform rods, nonmotile, nonsporulating, forming indol and $H_2S$, and producing acid from carbohydrates not in excess of pH 6.0.

*Anaerobic vibrios.* Included tentatively under this head are all strictly anaerobic gram-negative motile bacilli among these cultures. Morphologically, there are several types, including the typical curved or comma-shaped rods with one polar or subpolar flagellum; larger curved and spiral forms ("spirilla"), and small motile straight rods with rounded or tapered ends on which flagella have not been seen.

*Bacteroides.* These are strictly anaerobic gram-negative, nonmotile, nonsporulating rods.

*Leptotrichia.* The characteristics of these organisms conform with those given by Thjøtta, Hartmann, and Bøe[46] and Bøe.[3] They are large granular rods or unbranched filaments, gram-positive in young cultures but easily decolorized, anaerobic or microaerophilic, not producing indol or $H_2S$. They produce translucent rough "medusa-head" colonies on streaked plates. In this work they were isolated frequently from AFK cup plates, in which they were found in a distinctly fibrous outgrowth from the central area of mixed growth, easily distinguishable from the more homogeneous hazes of spirochetal growth. Dark-field preparations of such cultures often showed regularly intertwined "rope strands" of long filaments (Fig. 1).

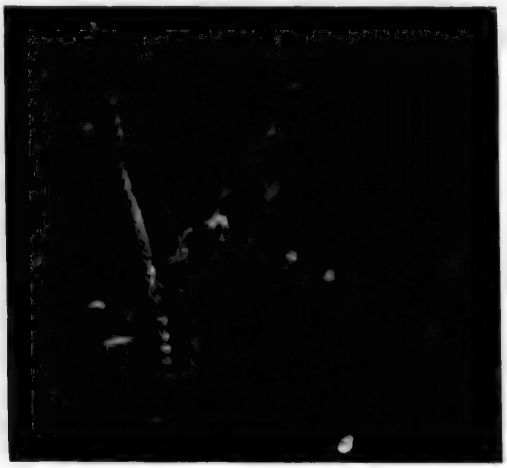

Fig. 1.—Filaments of Leptotrichia, including twisted strands. From a culture in an AFK spirochete plate. Dark-field illumination. ×1000.

*Actinomyces.* The characteristics of these organisms have been given by Rosebury, Epps, and Clark.[36] They are gram-positive rods growing as a branched mycelium, preferably under anaerobic conditions, tending easily to fragment into "twig" forms. They form opaque, white, heaped-up, more or less rough colonies on streaked plates, grow only below the surface of aerobic dextrose agar shake cultures, and generally appear as crumblike masses at the bottom of aerobic dextrose broth cultures.

*Anaerobic streptococci.* Gram-positive streptococci, otherwise variable in morphology from strain to strain, growing only under anaerobic conditions.

*Veillonella.* Strictly anaerobic small gram-negative* cocci growing in staphylococcus-like clusters.

*Anaerobic gram-positive bacilli.* This is a miscellaneous category of anaerobic organisms not conforming with the characteristics of Leptotrichia or Actinomyces. All cultures found in this work were nonmotile and nonsporulating, but otherwise heterogeneous.

*Anaerobic gram-negative threads.* This group comprises five strains with consistent properties that do not seem to have been described previously, although they conform in some respects with organisms described by Henriksen.[12] The latter's strains were, however, motile. Those found in this work were nonmotile, gram-negative, strictly anaerobic rods less than 0.3

*According to Foubert and Douglas[8] these organisms are gram-positive in very young cultures and should be classed with the anaerobic Micrococci.

mμ thick, so that they appeared single-contoured under dark-field illumination; they tended to grow as small, flat, depressed colonies on anaerobic blood agar plates.

Further data on some of these microorganisms are presented in later reports. It will be seen from Table III that their distribution among the different clinical entities was not such as to suggest that any of the types has special importance in one disease as compared with another. An exception to this statement may be made for the Actinomyces, isolation of which form exudate uncontaminated with the gingival flora as a whole is confirmatory of a clinical diagnosis of actinomycosis. It is known, however,[30] that these organisms can be isolated from the gingival flora in the absence of actinomycosis. Their low incidence in the present series of cultures may reflect the fact that less emphasis was placed on searching for them than for more characteristic members of the fusospirochetal flora.

The data in the last two columns of Table III show that certain of the groups that appear in cultures made directly from gingival scrapings were not found in cultures from fusospirochetal exudates. Although only 4 clinical cases were involved in the latter series, the data given represent the results of many trials on successive passage exudates, particularly on one guinea pig passage "strain" which has been used in the attempt to isolate its total flora. These findings are presented more fully elsewhere.[34, 35] It appears to be significant that Leptotrichia, Actinomyces, Veillonella, and the anaerobic gram-negative threads were not recovered from such exudates. Since these forms are likewise not seen in direct preparations from such exudates, it seems probable that they are not participating members of the pathogenic fusospirochetal flora.

## DISCUSSION

The data in this report constitute a survey of the gingival and periodontal flora of the human mouth, with particular reference to those portions of the total flora occurring under common pathologic conditions that can be separated from the whole (1) by inoculation of and passage through guinea pigs, and (2) by methods of anaerobic cultivation. The contents of the whole flora have been studied by direct microscopic examination of gingival scrapings under dark-field illumination; their pathogenicity has been compared for different gingival and periodontal diseases by guinea pig inoculation; and both the resulting experimental lesion exudates, after several passages, and the original scrapings have been subjected to preliminary analysis by anaerobic cultivation. Individual types of microorganisms observed by direct examination and in culture have been identified only provisionally, chiefly on morphologic grounds. The data make possible a tentative charting of the complex microbial association under study which may serve as a useful basis for further research in this field.

The dark-field data on 10 normal subjects support the commonly accepted view that the microorganisms found under pathological conditions are no more than overgrowths of those found normally, or, in other words, that the pathologic flora is indigenous to the mouth. Scrapings from 4 of these cases, and from a pool of the other 6 which was concentrated by centrifugation, were inoculated into guinea pigs. Three of the samples from individual mouths failed to elicit lesions. The fourth individual sample and the pool yielded small abscesses

which were not typical fusospirochetal responses, although spirochetes and other typical fusospirochetal organisms were found in their exudates in small numbers. Hemmens and Harrison[11] gave brief mention to a similar experiment performed by them, in which a more positive result was obtained after 5 to 7 transfers. These workers, however, found it necessary to use scorbutic guinea pigs to establish infection with scrapings from periodontitis, and observed beta hemolytic streptococci in guinea pig exudates after inoculation with scrapings from either pathologic or normal gingival tissues. It is possible that these two observations were related. The normal guinea pig is resistant to hemolytic streptococcus infections. In the present studies, normal animals were used exclusively; hemolytic streptococci have not been seen in their exudates. Since in these studies 32 per cent of 104 trials with pathologic scrapings failed to elicit typical fusospirochetal responses in guinea pigs, undue weight cannot be attached to negative findings in 5 trials with normal scrapings. It has not seemed profitable to undertake a larger series of guinea pig inoculations with normal material. The question of the occurrence of potentially pathogenic fusospirochetal organisms in the normal mouth would seem best answered after technics have been improved for isolating the individual types from such mouths and for recombining them to produce a pathogenic mixture. Meanwhile, in the absence of data showing differences in presence, kind, or virulence of organisms under normal as contrasted with pathologic conditions, the most economical hypothesis would seem to ascribe the difference to numbers only. It is assumed, in other words, that the fusospirochetal organisms occur in the normal mouth but in numbers too small to elicit infection in the guinea pig.

The responses of guinea pigs to inoculation with scrapings from the different gingival and periodontal infections make it plain that the fusospirochetal flora occurs in all these processes, with about equal frequency, and, allowing for technical errors in the animal inoculation test, probably uniformly. In the light of this finding, the differences in the flora seen by direct dark-field examination, particularly between Vincent's gingivitis and periodontoclasia, are of doubtful importance. Vincent's infection is the most acute and rapidly destructive of these diseases; periodontoclasia is the most chronic. Assuming that infection is no more than one aspect of the complex etiology of both diseases, the findings accord with the view that the infective process is relatively more significant and less complicated in the former disease. The microorganisms of Vincent's infection thus approximate more closely to the somewhat more limited flora of experimental guinea pig lesions, in which infection is uncomplicated by other etiological factors. The conclusion seems inescapable, nevertheless, that fusospirochetal infection is a factor in the etiology of all these diseases, including herpetic gingivostomatitis, in which the primary etiological agency is known to be infection with the virus of herpes.[6] In this disease, fusospirochetal infection seems to be "secondary" in the true sense, i.e., secondary to another infection. In the other diseases, so far as is known, the underlying causative agencies are not infective in nature.

The findings in anaerobic cultures indicate further that an essentially similar group of microorganisms occurs in the lesions of the different gingival

and periodontal diseases. The material studied included aspirated pus from 4 cases of suspected cervicofacial actinomycosis, in 3 of which the clinical diagnosis was confirmed by isolation of *Actinomyces israeli*. Dark-field examination of these exudates revealed a range of gingival organisms that included all the fusospirochetal types, but for the most part these organisms were found only in small numbers. The exudates were cultured particularly for anaerobic actinomycetes, and no attempt was made to isolate spirochetes from them. Since the evidence implicating *A. israeli* as the sole infective agent in actinomycosis seems adequate,[30] the presence of other organisms in the exudates may be ascribed to contamination of the lesions from the mouth, which is thought to be the source of the infection.

The absence in cultures made from guinea pig lesion exudates of certain types isolated from gingival scrapings directly is taken as evidence that the types in question are not members of the potentially pathogenic fusospirochetal flora. It seems noteworthy, however, that the flora of guinea pig exudates, even after repeated passage, contains a wider range of microorganisms than the four types implicated as contributing pathogens by the studies of Smith[43] and Proske and Sayers.[2] One "strain" of guinea pig passage exudate ("M.P.") persistently contained an aerobic greening streptococcus in addition to the types listed in Table III. More intensive studies of the cultures obtained from this guinea pig "strain," including their further identification and attempts to assess their pathogenic significance, are reported elsewhere.[34, 35]

## SUMMARY

Dark-field examination of suspensions in broth of gingival scrapings from 136 cases of gingival and periodontal disease and of 10 normal subjects points to a similar distribution of microorganisms throughout, with quantitative differences. The more characteristic fusospirochetal organisms (spirochetes, vibrios, and fusiform bacilli) were found in much smaller numbers in normal mouths than in any of the pathologic processes, in all of which they were prominent. Large numbers of these forms were found in a slightly higher proportion of cases in Vincent's gingivitis than in periodontoclasia or in marginal gingivitis. In periodontoclasia, and to a less degree in marginal gingivitis, organisms other than the fusospirochetal group (undifferentiated cocci, leptospiras, branched and unbranched filaments, and other types) were more commonly found or were found in larger numbers. The differences seemed too small to have diagnostic value.

Subcutaneous inoculation of guinea pigs with gingival scrapings from 40 subjects with various gingival or periodontal diseases resulted in typical fusospirochetal infection in from 75 to 92 per cent of trials. When these findings were combined with earlier data to give a total of 104 trials, the proportion of positive responses was found to be similar for the different clinical entities studied.

The distribution of types of microorganisms in anaerobic cultures from 35 cases of gingival or periodontal disease, made either directly from gingival scrapings or from lesion exudates after guinea pig passage, was again similar for the different diseases. Certain microorganisms obtained from gingival scrapings

were not found in cultures from guinea pig exudates. These microorganisms, Leptotrichia, Actinomyces, Veillonella, and an anaerobic gram-negative thread form hitherto apparently undescribed, seem in consequence to be unessential to the pathogenic fusospirochetal complex.

The data are considered to support the concept that a group of microorganisms indigenous to the mouth proliferates under pathologic conditions and contributes to gingival and periodontal disease.

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# THE PRODUCTION OF PERIODONTAL DISEASE IN THE HAMSTER AS RELATED TO DIET, COPROPHAGY, AND MAINTENANCE FACTORS

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## INTRODUCTION

IN VIEW of previous studies[1, 2] it seemed apparent that a prevalent form of experimentally induced periodontal disease in the hamster was associated with one or a combination of the following factors: (1) a high carbohydrate diet of fine consistency; (2) the absence of wood shavings (the presence of which might have aided in the self-cleansing of the teeth and periodontium during mastication —or supplied some unknown dietary factor); (3) the inability of animals maintained in wide mesh wire bottom cages to retain their feces and indulge in coprophagy—possibly resulting in some dietary deficiency.

This study was designed to delineate the effects of these factors and to demonstrate optimal conditions to be employed when it is desired to produce periodontal disease of this type in large numbers of young animals.

## METHODS

Seventy-five hamsters, approximately 32 days old, were distributed according to littermates into 5 groups, subdivided equally by sex. They were maintained in heavy wire screen cages of uniform size, nominal mesh opening approximately one-half inch. In spite of the use of the wide mesh screen, the hamster frequently grasps the fecal pellet as it emerges and eats it before it is lost from the cage, hence this method results only in a partial restriction of coprophagy. Beginning and end weights of all the animals were recorded.

*Maintenance Conditions:*

*Group I.*—Purina Laboratory Chow. Coprophagy limited, no bedding. On the basis of former studies,[2] very little disease was expected when the animals consumed the coarse commercial diet, but the restriction of coprophagy had not been tested under these conditions.

*Group II.*—CPD (caries-producing diet).* Coprophagy limited, no bedding.

*Group III.*—CPD. Fine mesh screen-bottom cages. No restriction of coprophagy, no bedding. This group served as a control to Group II in respect to the coprophagy factor.

*Group IV.*—CPD. Fine mesh screen-bottom cages. Coprophagy unrestricted. Shavings for bedding.

*Group V.*—CPD. Coprophagy limited. Shavings rationed daily—not for bedding.

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After approximately 98 days of this regimen, the animals were sacrificed and the molar areas of the wet jaws were examined under magnifications up to 30×. The condition of the gingival tissues was recorded on the caries scoring sheet advocated by Keyes,[3] and extent of gingival lesions was drawn and the area involved was colored red. Then a purely subjective impression of severity and extent of disease was recorded on a scale of 0 to 3. After all animals were scored, the following method of evaluation was used:

The "Number of Teeth Involved" indicated those in contact with one or more periodontal lesions in the upper and lower jaws. Based on an arbitrary choice of 10 periodontal areas for each quadrant (3 buccal, 1 opposite each molar; 3 lingual; the immediate area next to the mesial of the first and distal of the third molar; and 2 interproximal areas) the number of "Areas Involved" was recorded for the upper and lower jaws. The figures were added and averaged according to group and sex, and a frequency of occurrence of lesions of particular areas was established.

Upon autopsy, the gastric contents of 20 animals, 2 males and 2 females of each group of near-perfect littermate distribution, were examined grossly and the following tissues were prepared in paraffin for histologic study. The maxillae were cut in buccolingual sections, attempting to cut the first, second, and third molars of each animal. The molars of one mandibular quadrant of each animal were sectioned mesiodistally. Cross sections of each esophagus were made at the region of the thyroid gland.

## FINDINGS

Sixty-seven of the 75 animals used lived the full term of the experiment. The stomachs of the first 20 animals revealed no trichobezoar, contrary to expectations based on findings in cotton rats which were raised on low cellulose content diets.[4]

Fig. 1 is representative of the findings in the esophagi of the animals in each group. Note the dense keratinous layer. No mucous glands were seen in these sections. This parallels the findings of Ranvier[5] who found none in the rabbit, guinea pig, and rat, but located many in the submucous coat of the dog. Goetsch stated that ". . . the epithelium shows a very definite and constant relation to the character of the food, and it is in the epithelium that we find the esophagus undergoing specialization in those animals which live on coarse vegetable food."[6] Apparently, the degree of hornification of the epithelium of the hamster esophagus is a characteristic unalterable by the range of dietary consistency employed in this study for this length of time. In general, the keratin layer of the palatal epithelium appeared to be thinner than that of the esophageal epithelium. It was more compact, and had less tendency to distort as a result of the histologic preparations. There were no consistent differences in appearance of this structure in the animals of any one group, nor was there any apparent difference due to the sex of the animals. There was no significant difference in the beginning weights, and weight gain of the 5 groups. No con-

*Sucrose (4× confectionery sugar) 15 per cent; corn starch, 20 per cent; whole powdered milk, 30 per cent; whole wheat flour, 30 per cent; alfalfa, 4 per cent; NaCl, 1 per cent.

sistent sex difference in weight was apparent. The averages of the beginning weights of the 5 groups ranged from 48 to 61 grams. The averages of the end weights were 87, 88, 91, 91, and 89, Groups I to V, respectively. The presence or absence of shavings or unrestricted coprophagy had no effect on the weight gain.

*Periodontal Findings—Macroscopic:*

The animals of Group I, which had subsisted on the laboratory chow and were maintained on the wide mesh wire-bottom cages without shavings, presented rather characteristic findings apparently related to the fibrous consistency of their diet. Three males and 1 female showed no periodontal lesions discernible under the dissecting microscope. In a few instances slight recession was seen without any apparent cause. Each of the other 10 presented from 1 to 3 localized areas of periodontal destruction. These, almost invariably, were the interproximal spaces of the upper jaw. The typical lesion consisted of the packing of fibrous material and hair into this area, with resulting gingival trauma and recession. The severity of periodontal disease, when present, was always scored number one in these animals, since there was no evidence of generalized destruction, and because the lower jaws were so nearly free from lesions.

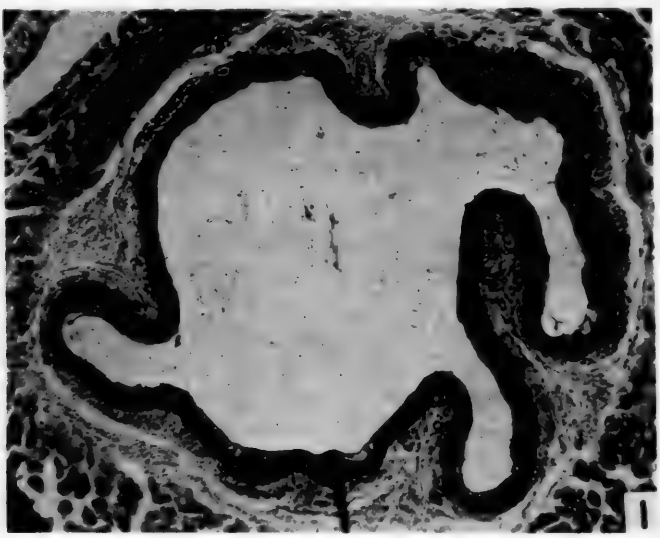

Fig. 1.—A Cross section of esophagus, at level of thyroid gland of a Group I female. Representative of findings in animals of all groups. Note dense keratinous layer and absence of mucous glands.

In contrast to those observed in Group I, the lesions found in the animals of Groups II through V were quite different. The typical lesions associated with the soft, fine, high carbohydrate diet are pictured in Figs. 2 and 3. Note the periodontal condition particularly on the palatal surface of the upper left first and second molars. The tightly-packed, whitish, fine-grain subgingival accumulations were found only in those animals fed the caries-producing diet.

By far, the areas most often affected were the lingual and mesial surfaces of the lower first molars; second, the palatal, and third, the buccal surface of

the upper first molars. The second molars were affected less and the third molars the least.

The animals of Groups II and III presented no interproximal lesions comparable to those observed in Group I. However, 4 animals of Group IV, which had been maintained constantly on shavings, presented such lesions with pieces of shavings packed down between the upper molars. One animal from Group V presented such a lesion. It is of interest to note that this potential predisposing factor to periodontal destruction did not increase the over-all incidence of periodontal disease in these animals.

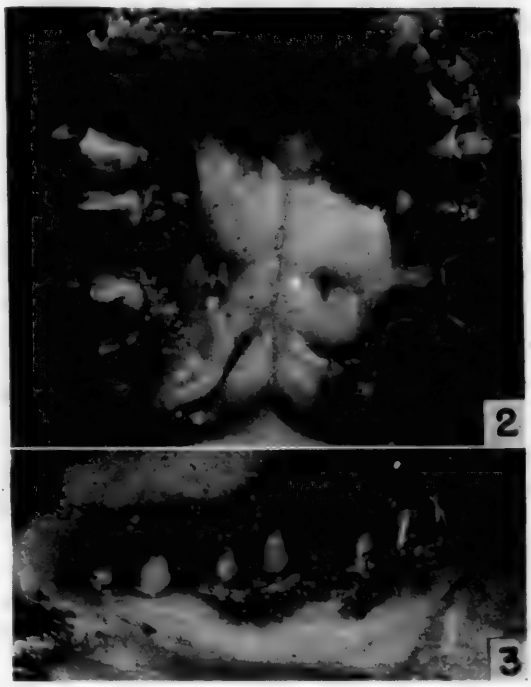


Fig. 2.—Maxilla, Group II female. Left third molar was destroyed by caries. Note large accumulations on palatal surfaces of left first and second molars.

Fig. 3.—Lower left molars of same animal (Fig. 2). Tightly packed, whitish, fine-grain gingival accumulations were common in animals of Groups II-V inclusive.

The periodontal disease scores of the animals of Groups II to V were considerably higher than those of Group I (Table I). The average number of teeth involved per male and female of Group I was 2.71, as compared to similar figures ranging from 7.0 (Group IV) to 8.6 (Group V). This low figure for Group I existed in spite of the fact that the involved areas were generally interproximal spaces and thus involved 2 teeth. The average number of areas involved in males and females of Group I, upper and lower jaws, respectively, was 1.5 and 0.2. These figures were much lower than those ranging from 5.2 and 5.5 (Group IV) to 8.3 and 6.6 (Group V). The average degree of severity for Group I was 0.71, whereas the degree ranged from 1.64 (Group IV) to 2.16 (Group III) in the other groups.

It might appear at first that Group V was the most seriously afflicted, but both severity and the average number of lower areas involved check closely with similar figures of Group III. We are led to believe that there is no consistent significant difference in the degree or extent of periodontal disease in Groups II to V.

Preliminary inspection suggests that the females were more afflicted than the males; however, there are several exceptions. If a sex difference in susceptibility to periodontal disease does exist, it is not apparent from this study.

TABLE I

TABULATION OF PERIODONTAL DISEASE

| GROUPS* | NUMBER TEETH INVOLVED | AVERAGE | AREAS INVOLVED | | AVERAGE | | DEGREE SEVERITY | |
|---|---|---|---|---|---|---|---|---|
| | | | UPPER | LOWER | UPPER | LOWER | TOTAL | AVERAGE |
| Group I | | | | | | | | |
| 7 ♂ | 12 | 1.71 | 8 | 2 | 1.1 | 0.1 | 6 | 0.85 |
| 7 ♀ | 26 | 3.71 | 13 | 2 | 1.8 | 0.1 | 4 | 0.57 |
| 14 ♂ + ♀ | 38 | 2.71 | 21 | 4 | 1.5 | 0.2 | 10 | 0.71 |
| Group II | | | | | | | | |
| 7 ♂ | 48 | 6.80 | 43 | 31 | 6.1 | 4.4 | 12 | 1.70 |
| 5 ♀ | 41 | 8.20 | 32 | 33 | 6.4 | 6.6 | 8 | 1.60 |
| 12 ♂ + ♀ | 89 | 7.41 | 75 | 64 | 6.2 | 5.3 | 20 | 1.66 |
| Group III | | | | | | | | |
| 5 ♂ | 38 | 7.60 | 30 | 32 | 6.0 | 6.4 | 12 | 2.40 |
| 7 ♀ | 57 | 8.10 | 45 | 48 | 6.4 | 6.8 | 14 | 2.00 |
| 12 ♂ + ♀ | 95 | 7.90 | 75 | 80 | 6.2 | 6.6 | 26 | 2.16 |
| Group IV | | | | | | | | |
| 7 ♂ | 50 | 7.10 | 33 | 45 | 4.7 | 6.4 | 12 | 1.71 |
| 7 ♀ | 49 | 7.00 | 41 | 32 | 5.8 | 4.5 | 11 | 1.57 |
| 14 ♂ + ♀ | 99 | 7.00 | 74 | 77 | 5.2 | 5.5 | 23 | 1.64 |
| Group V | | | | | | | | |
| 8 ♂ | 65 | 8.10 | 56 | 48 | 7.0 | 6.0 | 18 | 2.28 |
| 7 ♀ | 64 | 9.10 | 69 | 51 | 9.8 | 7.3 | 14 | 1.96 |
| 15 ♂ + ♀ | 129 | 8.60 | 125 | 99 | 8.3 | 6.6 | 32 | 2.13 |

Group I—chow, no bedding; Group II—CPD (caries-producing diet) no bedding; Group III—CPD, fine screen cage bottom, no bedding; Group IV—CPD, fine screen, shavings for bedding; Group V—CPD, no bedding, rationed shavings.

Although the total number of areas involved in the upper jaws is 740 and that of the lower jaws is 648, it is not felt that, by this method of scoring, a significant difference in incidence between the two jaws has been demonstrated, except in the case of Group I, in which practically all the lesions (interproximal) were found in the upper jaw.

*Periodontal Findings—Microscopic:*

In general, the microscopic findings in the jaws support those of the gross examination. No periodontal destruction was found in 1 animal of Group I, and very little was seen in the other 3 animals of this group which were studied in this manner. Small microscopic subgingival deposits of a granular basophilic material were seen in the latter.

The sections from animals of Group II through V showed marked destruction with accompanying loose, granular basophilic subgingival accumulations (Fig. 4). Note the loss of periodontal bone and the massive destruction of palatal

tissue. It is of interest to note that the same deposit which appears to bear such a close relationship to the periodontal loss is apparently intimately allied with the carious process beginning on the palatal surface of the root of the molar on the right (Fig. 5).

In most cases the mesiodistal sections of the lower molars did not reveal such periodontal destruction except on the mesial of the first molar.

Fig. 4.—Buccolingual section of the maxillae of Group II male. Note massive destruction of palatal tissues in presence of large, basophilic granular subgingival deposits.

Fig. 5.—Magnified view of area of Fig. 4. Note presence of periodontal and dental lesion associated with the deposit. Secondary dentin is seen beneath cavity.

## DISCUSSION

This study clearly reveals the fact that practically all hamsters of this colony are susceptible to some form of periodontal destruction. It is further brought out that there are at least two forms of periodontal disease which may be induced. A coarse fibrous diet of laboratory chow (and/or access to wood

shavings) results in an interproximal impaction of fibrous food, hair, and cellulose, most common for some unexplained reason in the upper jaw. The second form of disease observed was closely associated with a soft, fine, high carbohydrate diet. This regimen resulted in the deposition of a white, soft, granular, basophilic mass in the gingival crevices. It is suggested that this deposit may be related to the gummy consistency of the moistened diet. In these animals, the interproximal areas are relatively free of this deposit and resultant destruction This may be due to the high epithelial attachment on this surface of the tooth, and to the fact that the rather broad, tight contact points prevent the forcing of this material below the gingivae. This hypothesis is supported by the fact that the mesial surfaces of the first molars are so affected, and there is no approximating tooth at this point. It is well to remember that the epithelial attachment is of a slightly different nature in the interproximal areas than that on the "noncontactual" surfaces of the teeth.[1]

The microscopic deposits of basophilic material seen in the histologic preparations of Group I animals were not observed grossly. The material was more densely stained than that found in the other groups, and no caries was found in association with it.

The periodontal lesions, as seen in all animals, seem to bear a marked relationship to a pressure type of destruction due to the subgingival accumulations. No deep, narrow pockets were seen. These macroscopic deposits observed in the hamster did not appear to be a type of calculus similar to that seen in human beings because they were intimately associated with the carious process, and no firmly attached concretions were seen while the jaws were in the fresh wet state. King and Gimson[7] have reported that "tartar," "calculus," and "concretions" appeared in hamsters after 19 weeks on a diet of somewhat similar consistency but of varied constituents. The frequency of occurrence of lesions was somewhat different from that reported here but dietary and time variables may have accounted for that fact. They emphasized the proximity of the most frequent lesions encountered—on the buccal surfaces of the upper first molars and the lingual surfaces of the lower first molars—to the salivary gland ducts of the two jaws. This observation is not born out in this study.

King[8] also has reported "salivary calculus" in the hamster and excavation of tooth roots ". . . largely due to destruction of cementum and dentin by the products of the inflammation set up in the periodontal membrane just ahead of the proliferating calculus." The diet used in this study again was somewhat similar in consistency but varied greatly in constituents, notably the absence of sugar, when compared to the diet used in this study. In subsequent work King[9] did use a high sugar diet supplemented with carrots and cabbage, and further described resulting "primary cavitation of the cementum and dentine of the tooth roots." He found no coronal caries in these animals and was led to believe that: "Later disease phases include extension of the cavities into the pulp, followed by breakdown of the coronal dentine and enamel, but disintegration of the tooth crown, with the appearance of caries-like cavities, is essentially secondary to the initial root excavations which themselves result from the periodontal disturbances." In light of the variables, such as duration of experi-

mental periods, diets employed, and possible strain variations in the animals used, it is not possible to compare closely the above works of King and the study here reported.

It would seem that the "plaque" material mentioned by Keyes[10] and Keyes and Likins[11] in connection with hamster caries and periodontal disease is the same as this deposit which occurred invariably with periodontal disease induced by a high carbohydrate diet (when shavings or other coarse matter were not involved in the lesions).

In view of the results, it would seem that the regimen employed with Group II would lend itself best to the production of generalized periodontal disease in large numbers of animals for studies, for example, of the effects on the periodontium of deficiency states or preventive agents in the presence of a predisposing factor, the gingival debris, without resorting to more artificially induced irritation.

## CONCLUSIONS

The majority of hamsters maintained on a coarse laboratory chow diet over a period of about 98 days developed mild periodontal disease. This was usually in the form of interproximal lesions in the upper jaws, associated with impaction of the fibrous food and hair between the teeth. A fine, soft, high-carbohydrate diet fed over the same period of time resulted in a moderate to severe, and more or less generalized form of periodontal disease in practically all animals so maintained. No sex difference in incidence was apparent.

Partial restriction of coprophagy and the presence or absence of wood shavings did not influence the over-all incidence of periodontal disease or weight gain in these animals. Hornification of palatal and esophageal mucosa did not vary with the type of diet or maintenance conditions.

Dental caries and periodontal disease occurred in close association with one another, and evidence indicated that the subgingival carious plaque material and the periodontal accumulations were the same.

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# EFFECT OF CERTAIN ABRASIVE MATERIALS ON TOOTH ENAMEL

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A WIDE variety of prophylactic materials and technics are being employed in the dental office for the removal of stain and calculus. Unfortunately, little research has been done concerning the effect of these materials upon the enamel and dentin surfaces. It is quite possible that certain types of materials and technics now being employed are detrimental to the tooth surface. Such deleterious effects could occur not only from excessive abrasion and resulting tooth loss but also from production of a rough, dull surface which might accumulate stain and debris more rapidly. If possible, one would desire a prophylactic agent in which the material will have adequate cleansing power, yet leave a surface which is, or can be, easily polished. A polished surface will be better esthetically and will remain cleaner longer. Certainly before a definite universal procedure for cleansing teeth can be evolved, the effects of these abrasive materials and prophylactic technics need the closest of study.

Much research has been done by many investigators in regard to the action of dentifrices upon the tooth surface.[1-6] These studies have included weight and micrometer measurements of tooth loss as well as visual and microscopic examination of surface changes. None of these investigators, however, included the typical prophylactic materials and technics commonly employed, nor have they made use of accurate testing apparatus for detecting luster changes on the tooth surface. The purpose, therefore, of this study was to (1) measure accurately the amount of enamel removed by various materials and technics, (2) study the effects of the cleaning agent upon the tooth surface, both by luster measurements and microscopic examination, and (3) determine the rapidity with which the luster can be restored to the tooth surface with a polishing agent. As nearly as possible, the testing procedures closely paralleled the clinical technics employed with these prophylactic materials.

*Enamel Loss*

The measurement of surface loss was based upon a technic of focusing on the tooth surface by means of a Bausch & Lomb metallographic microscope, reading the scale on the fine adjustment knob, treating the surface with the cleaning agent, refocusing, and then computing the surface loss in microns. This technic is a modification of that used by Manly.[4]

Sound, freshly extracted upper incisor teeth were used throughout this study. Teeth were not allowed to remain out of water longer than absolutely necessary. The labial crown surface of the tooth was ground and polished in order to prepare a small, flat surface about 4 mm. in diameter. This flat area was necessary for obtaining an even and accurate focus on the tooth surface.

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The tooth was then mounted with compound on a specially prepared glass disk which fitted into the stage aperture of the microscope. The disk and stage were marked so that the specimen could be removed and replaced in exactly the same position. Very pertinent to the success of this study was the necessity of being able to replace the specimen in exactly the same position each time; thus, any change in focus and the resulting reading of the vernier scale could be attributed solely to the effect of the abrasive and not to any change in the specimen position. Repeated tests by removing and replacing the glass disk, with the tooth attached, indicated the maximum error to be one division on the fine adjustment scale, which was equivalent to 2.5 microns.

After the tooth surface on the test specimen had been brought into focus, a reading was taken. The tooth was then treated with the particular prophylactic material and replaced on the stage aperture. The surface was brought into focus again and the amount of enamel removed determined.

**Enamel Loss With Airbrasive**
**(nozzle height and treatment time variable)**

| Distance from nozzle to tooth | Treatment time (in seconds) | Average loss (in microns) |
|---|---|---|
| 1 mm | 5<br>10<br>15 | 35<br>69<br>106 |
| 5 mm | 5<br>10<br>15 | 15<br>27<br>40 |
| 10 mm | 5<br>10<br>15 | 7<br>9<br>11 |
| 15 mm | 5<br>10<br>15 | 3<br>5<br>6 |

Table I.

One of the most recent technics for removing tooth stain and calculus is the use of the Airdent which employs a stream of abrasive directed against the tooth surface by carbon dioxide pressure.[7] It was felt that this technic warranted investigation, particularly in regard to the enamel loss as related to the treatment period and the distance of the nozzle from the tooth surface. In order that this distance from the nozzle to the tooth surface could be accuraely controlled, a special piece of apparatus was employed. The nozzle was attached to the end of a vertical arm which was calibrated in millimeters so that the nozzle end could be raised an exact distance from the nozzle to the tooth surface. The nozzle was at right angles to the tooth. The arm was then locked in this position during the test. The application period was timed by means of a stop watch. The recommended nozzle pressure of 40 pounds per square inch was used and the prophylactic cleaning abrasive, dolomite, was employed throughout.

The average enamel loss at nozzle distances ranging from 1 mm. to 15 mm. and for periods of time ranging from 5 to 15 seconds is seen in Table I. Each figure is the average of at least 3 individual tests. Since the maximum experimental error for this technic is 2.5 microns, all changes are above this error.

When the nozzle is held close to the tooth surface, 1 mm., the enamel loss can be quite great as the application time is increased. At the end of 15 seconds of treatment, the average enamel loss is 106 microns. As the distance of the nozzle from the tooth surface is increased, the enamel loss becomes progressively less. At a distance of 15 mm., the loss is only 6 microns after 15 seconds of application. It can be noted from these data that the enamel loss is not a direct function of application time. For example, when the nozzle is held at 10 mm. from the surface, after 5 seconds of treatment the loss is 7 microns but at 15 seconds the loss is only 11 microns. This variation may be accounted for by numerous factors, such as the slightly uneven flow of the abrasive and different areas of hardness in the enamel. It can be seen from these tests that this prophylactic abrasive does remove the enamel, particularly when the nozzle is held at right angles close to the tooth surface for long periods of time.

It is difficult to interpret these results from the clinical standpoint. Undoubtedly, however, to minimize loss of tooth structure, care must be exercised in the use of this technic. The abrasive does remove stain and calculus from the surface rapidly and it is not necessary to hold the nozzle too close to the tooth surface. Stain can be removed readily at 15 mm. distance but calculus requires longer treatment time or closer position of the nozzle. Generally, however, it is not necessary to hold the nozzle close to the surface for long periods of time. Certainly, the stream of abrasive should be stopped immediately after the last stain or calculus has been removed. More work needs to be done in regard to the effect of this technic on cementum and dentin where the structure loss might be much greater.

The enamel loss with 2 other typical abrasives, flour and fine pumice, was studied in this same manner. Denseo screw-shank Crescent rubber polishing cup was used with a slurry of the particular abrasive. The cup was revolved at a speed of 5,826 r.p.m. Flour of pumice was applied for periods of time ranging from 5 to 25 seconds with no measurable amount of enamel being removed. When a slurry of S. S. White fine pumice was employed, the maximum loss after 25 seconds was only 4 microns. It thus appears from these data that the enamel loss with flour or fine pumice is much less than with the airbrasive, even when the nozzle was held at 10 or 15 mm. from the surface.

It must be pointed out that the action of the airbrasive apparently produces a rather even removal of enamel over the entire area which it strikes while the pumice produces scattered scratching and not, therefore, a general leveling of the enamel surface. Attempts were made to determine the depths of the scratches produced but the data were not believed to be above the experimental error. Of course, as the pumice application time is increased, the greater is the number of scratches produced and therefore a more general removal of tooth surface.

### EFFECT ON LUSTER

In order to detect minute changes in the polish on a tooth surface, as affected by prophylactic materials, a specially designed reflectometer was employed. This apparatus, designed and secured through the courtesy of the Pepsodent Division of Lever Brothers Company, can be seen in Fig. 1. Constant voltage for the light source was obtained by means of a 57-plate, 6-volt storage battery and controlled by a rheostat. A beam of light, 1 mm. in diameter, enclosed in an aluminum housing (*d*), strikes the specimen (*b*) and is reflected into the photoelectric cell (*c*). This in turn activates a galvanometer. The smoother the tooth surface, the greater is the reflected light, the less is the diffuse light and consequently the higher is the galvanometer reading.

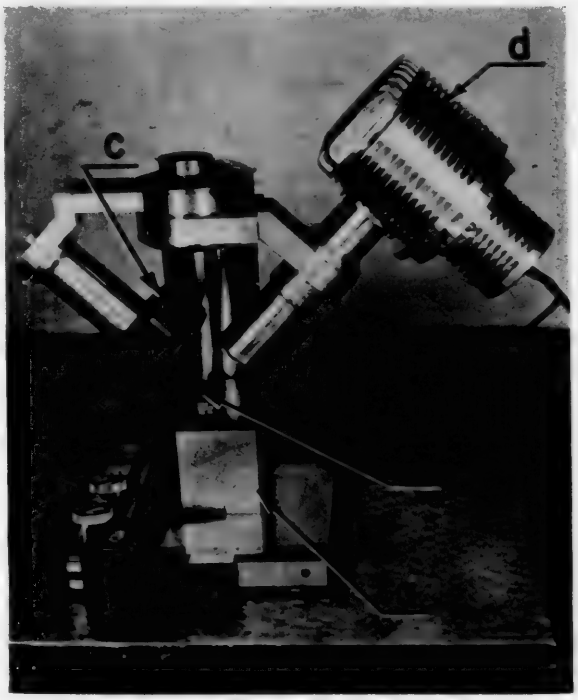


Fig. 1.—Reflectometer used to measure luster (secured through the courtesy of Pepsodent Division of Lever Brothers Company).

The movable stage (*a*) upon which the specimen is secured is calibrated so that the tooth can be placed in exactly the same position for each reading. However, in order to minimize any error due to a slight change in specimen position, the entire tooth area is traversed and the high reading taken as the maximum luster. In this photograph is shown the black Carrara glass standard (*b*) which is used for adjusting the light beam to the same intensity before each measurement.

Galvanometer readings may vary from a low of 20 units on a dull abraded tooth surface to a high of 75 on a highly polished surface. Thus it is possible to have a maximum variation of approximately 55 units on a tooth surface treated by different materials. When the apparatus is properly aligned, the experimental error for this technic is ± 1.5 units.

The effect of flour of pumice upon the luster of a highly polished tooth surface can be seen in Table II. The tooth was first raised to its maximum luster by use of levigated alumina. A slurry of flour of pumice was then applied by means of a rubber cup. In Column 1 is shown the average loss, in luster units, after 5, 10, and 15 seconds of application. These figures again represent the average of at least 3 individual tests. The flour of pumice had only a slight dulling effect and increasing the treatment period did not lower the luster. The rapidity with which this somewhat duller surface can be brought back to its original high polish can be seen in Columns 2 and 3. In Column 2 is shown the average change, from the original, after these same surfaces had been polished for 5 seconds with a slurry of levigated alumina. Levigated alumina was selected because of its even and rapid polishing action. When the pumice treatment time is 15 seconds, the average luster is 3.7 units lower than the original reading. After 5 seconds of polishing with levigated alumina, the luster is still 2.0 units lower than the original. However, after an additional 5 seconds of polishing, making a total time of 10 seconds (Column 3), the luster does return to the original.

Flour Of Pumice (S.S. White)

| Average Change In Luster From Original | | |
|---|---|---|
| 5 sec. pumice | 5 sec. polish* | 10 sec. polish* |
| -3.2 | - .4 | 0 |
| 10 sec. pumice | | |
| -3.9 | - .6 | +.4 |
| 15 sec. pumice | | |
| -3.7 | -2.0 | +.8 |

*Polished with rubber cup, levigated alumina

Table II.

Fine Pumice (S.S. White)

| Average Change In Luster From Original | | |
|---|---|---|
| 5 sec. pumice | 5 sec. polish* | 10 sec. polish* |
| -7.0 | - .7 | +.4 |
| 10 sec. pumice | | |
| -6.0 | -3.7 | -.4 |
| 15 sec. pumice | | |
| -6.0 | -3.0 | +.3 |

*Polished with rubber cup, levigated alumina

Table III.

The effect of S. S. White fine pumice is shown in Table III. With this abrasive the luster is lowered more than when flour of pumice is employed, and it is more difficult to restore the tooth to its original luster. For example, after 10 seconds of pumice application, the luster is 6 units lower than the original and it is still 3.7 units below the original following 5 seconds of levigated alumina polishing. Again, however, it can be seen that the effect of the abrasive, even after 15 seconds of continuous application, can be removed and the original luster restored after 10 seconds of polishing with levigated alumina.

Data were also secured relative to the effect of flour and fine pumice when a Crescent brush was employed instead of a rubber cup. In Table IV are

shown the average luster changes with flour of pumice and it can be seen that the luster is lowered more with the brush than with the rubber cup. For example, after 5 seconds of application with a rubber cup the luster was lowered 3.2 units as compared to 8.7 units when the brush was used. However, the luster was readily restored with polishing by levigated lumina. In Table V the

Fine Pumice (S.S White)

| Average Change In Luster From Original | | |
|---|---|---|
| 5 sec. pumice | 5 sec. polish* | 10 sec. polish* |
| -7.3 | -1.0 | -0.3 |
| 10 sec. pumice | | |
| -8.3 | 0 | 0 |
| 15 sec. pumice | | |
| -8.5 | -0.5 | 0 |

*Polished with rubber cup, levigated alumina

Table IV.

Flour Of Pumice (S.S. White)

| Average Change In Luster From Original | | |
|---|---|---|
| 5 sec. pumice | 5 sec. polish* | 10 sec. polish* |
| -8.7 | -1.7 | 0 |
| 10 sec. pumice | | |
| -8.7 | -0.7 | 0 |
| 15 sec. pumice | | |
| -10.0 | -2.5 | -0.5 |

*Polished with rubber cup, levigated alumina

Table V.

data are obtained by using S. S. White fine pumice applied with a brush. Again the luster is slightly lower than when a rubber cup was used. Results from these tests indicate that although the luster is lowered somewhat more with a brush, there is no great difference between the action of a rubber cup and a brush with a particular abrasive.

Effect Of Airbrasive
(nozzle height variable)

| Distance from nozzle to tooth | Average Change In Luster From Original | | |
|---|---|---|---|
| | Airbrasive | 5 sec. polish* | 10 sec. polish* |
| 5 mm | -48 | -5.5 | - .5 |
| 10 mm | -48 | -4.0 | - 1.0 |
| 15 mm | -46 | -3.0 | - 1.0 |

*Polished with rubber cup, levigated alumina

Table VI.

Table VI shows the average change in luster following use of the airbrasive when the nozzle was held at 5, 10, and 15 mm. from the tooth surface. The application time was 5 seconds. The dulling effect of the airbrasive was great, lowering the luster 48 units when the nozzle was held 5 mm. from the tooth surface. This compares with a net change of only 6 units when fine pumice was used. It can be seen from these data that the dulling of the surface was not altered appreciably by changing the distance of the nozzle from the tooth surface. It is interesting to note, however, that the effects of the airbrasive can be readily removed by means of a polishing agent. For example, when the nozzle

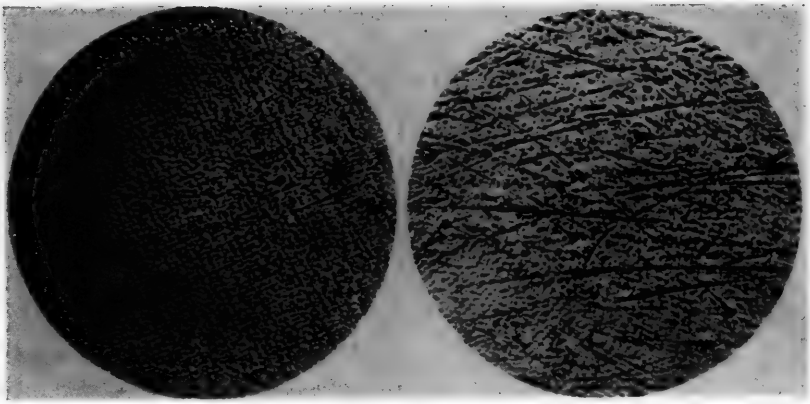

Fig. 2. Fig. 3.

Fig. 2.—Original polished enamel surface (200×).
Fig. 3.—Surface after flour of pumice application (200×).

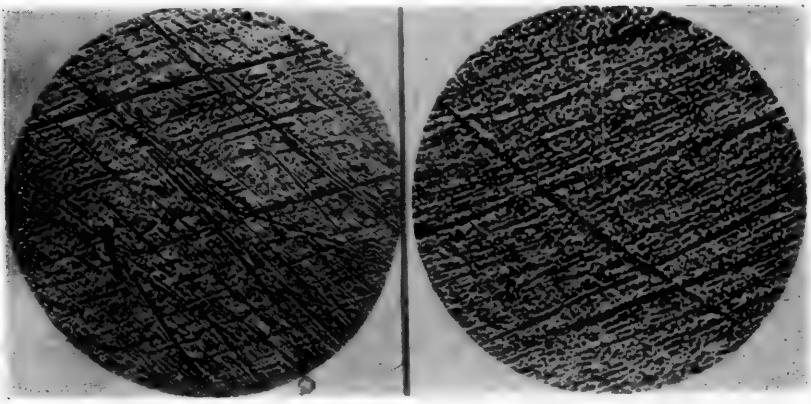

Fig. 4. Fig. 5.

Fig. 4.—Surface after fine pumice application (200×).
Fig. 5.—Surface after medium pumice application (200×).

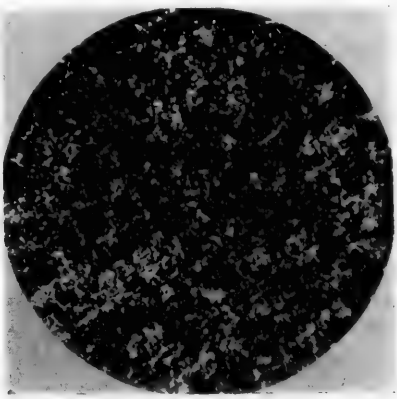

Fig. 6.—Effect of airbrasive on enamel surface (200×).

was held 5 mm. from the tooth surface, the luster loss was 48 units. However, after 5 seconds of levigated alumina polishing, the luster was raised until it was only 5.5 units below the original value. This is approximately an 80 per cent luster recovery. After another 5 seconds of polishing, the luster is back to the original (0.5 being within experimental error). Thus these data indicate that although the airbrasive does have a marked dulling effect on the tooth surface, the original luster can be readily restored by the use of a polishing agent.

The enamel surfaces are being studied microscopically, both before and after use of each prophylactic material. In Fig. 2 can be seen the polished tooth surface before the application of a cleaning agent. Fig. 3 shows this same surface after cleaning for 5 seconds with a slurry of flour of pumice. Some slight scratching and pitting have taken place but not enough to alter the luster appreciably. In Fig. 4 is the polished surface after the application of fine pumice for 5 seconds and the abrasion has become considerably greater, with a still more marked effect evident in Fig. 5, where a medium grit pumice slurry was employed.

Naturally, this scratching does tend to lower the luster of the tooth surface as shown by the previous data. Fig. 6, however, shows the action of the airbrasive in which the surface appears very dark due to the pitting. The luster on such a surface would, of course, be much lower than when pumice is employed since the roughness covers the entire area and is not scattered as are the scratches induced from pumice. No attempt has been made to compare the depth of these pits with that of the scratches caused from pumice. Apparently, however, these pits are not of great depth since the polish can be restored quite readily to this surface, as shown by the previous data and by microscopic examinations of the surfaces. There is no doubt that a polishing agent must be used after the airbrasive in order to eliminate this rough surface, thereby raising the luster and making the surface less susceptible to the accumulation of stain and debris.

## CONCLUSIONS

This is a progress report and much research remains to be done in regard to the action of these and various other prophylactic materials and technics on the tooth surface. For example, the enamel loss as affected by the angle at which the airbrasive nozzle is held should be investigated as well as the influence of carbon dioxide pressure and rate of flow of abrasive. The technics described in this paper do, however, make possible accurate measurement of surface losses and luster changes.

The data obtained indicate the general enamel loss to be much greater with the airbrasive than with flour of pumice or fine pumice. This enamel loss, however, may not be of practical significance when the nozzle is not held close to the tooth surface and the application time is not excessive. The luster of the tooth is greatly lowered by the airbrasive with only a slight effect induced with flour or fine pumice. The type of surface produced by the airbrasive is different

from that produced by pumice, but in all cases the original luster can be restored within 10 seconds by use of the polishing agent here employed.

The authors wish to acknowledge the capable assistance of M. L. Swartz and J. O. Stewart in this investigation.

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# DIMENSIONAL CHANGES IN DENTAL STONE AND PLASTER

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## INTRODUCTION

THIS report presents a method of determining the dimensional changes in gypsum dental products, which avoids the disadvantages of previous methods and which provides extreme sensitivity.

As part of the program of research on the gypsum products used in dentistry, a preliminary study was made of various physical properties. This study, which included strength, thermal expansion, setting expansion, and other related properties, soon made it apparent that very little was known about dimensional changes occurring in these products when they are subjected to the technics used in prosthetic dentistry. Inasmuch as the final accuracy of a restoration is controlled to a large extent by these changes in the investing materials, a program was set up to determine the dimensional changes which occur when plaster and stone are subjected to the conditions found in dental practice.

## PREVIOUS METHODS FOR DETERMINING DIMENSIONAL CHANGE

The methods which have been used by previous investigators, such as Coleman,[1] Taylor, Paffenbarger, and Sweeney,[2] Worner,[3] Docking and Donnison,[4] and others, to observe the dimensional changes of gypsum materials used in dentistry have been directed more toward obtaining reliable data on the physical change in linear dimensions of dental plaster, stone, and investments during the setting process than the changes caused by the processing of restorations. The determination of the thermal expansion of casting investments has been the subject of numerous investigations. The equipment used most extensively for thermal expansion determination of dental materials is the fused-quartz tube apparatus[5] which was developed to measure the thermal expansion of solids.

The methods used to observe length changes of gypsum after it has set have been rather crude and are not adaptable to observing the effect of various conditioning and processing technics. Basically, three methods have been used to measure these changes, with each having its characteristic disadvantage. Those methods that depend on a micrometer coming in contact with the surface of a plaster or stone sample are subject to error on repeated measurements because of the ease with which the surface is abraded or worn away. The methods which use index lines and a comparator microscope present practical difficulties in alignment and in getting suitable lines on the sample.[3] Methods for observing length changes by using a dial gauge in contact with a single specimen, which is not removed, restrict the number of specimens that can be measured conveniently. Also, it is sometimes desirable to maintain specimens under storage conditions which are impractical with the dial gauges.

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The necessity of developing a more suitable method for measuring plaster and stone specimens when subjected to conditions simulating those used in dentistry led, after several attempts, to the method to be described.

## PROPOSED METHOD OF MEASUREMENT

A method was developed which made use of the surface of three embedded metal spheres to determine a reference plane at one end of the specimen, and the surface of a single embedded sphere to determine a point on the other end. This practice avoids dimensional change due to wear in measurement and handling.

The change in length of the specimen is determined by observing the difference in dimensions developed between the reference plane and the point. This can be done by any one of several length measuring technics. It has been found that by comparing the length of the specimen with gauge blocks, by use of an Electrolimit Comparator (Pratt and Whitney, Hartford, Conn.), it is possible to make rapid measurements accurate to the order of 0.0001 inch or better. A sensitive dial gauge might also be used, but would require more care to achieve the same precision.

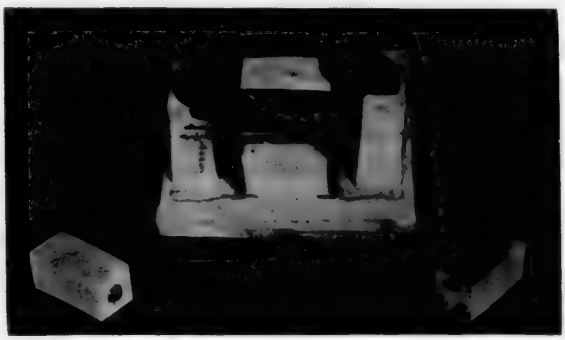

Fig. 1.—Mold for casting gypsum specimens.

The following is an outline of the method used in making observations on gypsum materials such as dental plaster and stone:

1. A form, which consisted of plastic elastomer sides (V-B-M) and metal ends, to mold the specimen (1″ × 1″ × 2½″) was prepared. The ends had countersunk holes into which were placed stainless steel balls. At one end the holes were spaced to form an equilateral triangle, at the other a single hole was centered. The metal plates were brass and the stainless ball bearings were held in the countersunk holes by means of permanent magnets (Fig. 1).

2. The gypsum to be measured was mixed and poured into the assembled mold to harden or set.

3. The mold is then disassembled after the gypsum has hardened and the specimen is removed.

4. The initial length of the specimen should be determined in the Electrolimit Standard External Comparator by placing the specimen in the comparator

and adjusting the instrument to read zero on the scale (Fig. 2). The scale is sensitive to 0.00005 inch per division.

5. The specimen is then removed from the comparator and gauge blocks are inserted to the nearest 0.001 inch, the difference being read on the scale of the instrument to the nearest 0.0001 inch. This gives a direct comparison to gauge blocks for each length measurement.

6. The specimen then can be exposed to any treatment such as drying, wetting, heating, or the curing cycle of a denture, and the measurement repeated as before.

7. The change in length is computed on the basis of the fiducial reading. The length of the specimen should be corrected for the size of the spheres. Corrections can be made for changes in room temperature, or the specimens can be brought to a constant temperature before measurement.

Fig. 2.—Electrolimit Comparator with specimen in position.

## APPLICATION OF METHOD

In order to clarify the use of the above method, the following example (Table I) is given for one specimen, of a series being used in an investigation of the effect of various treatments upon the dimensions of gypsum products. The complete results of this investigation will be reported later. This particular specimen was of R & R White Castone (Ransom and Randolph) and was subjected to a cyclic treatment involving alternate storage in air (63 per cent relative humidity, 25° C.) and in water (25° C.).

The mix was prepared by the standard technic used for dental stones in this investigation. A water powder ratio of 30:100 was used. The mix was hand spatulated for 15 seconds, placed in a Kerr Vacuum Investor (Kerr Manu-

facturing Co., Detroit, Mich.) for 30 seconds, and then vibrated into the mold. Three 3/8 inch and one 7/16 inch steel ball bearings were used on the ends of the specimen. To keep the balls from rotating, a small flat was ground on the embedded side of each.

TABLE I

EXAMPLE OF DATA AND CALCULATION
MATERIAL—CASTONE SPECIMEN NO. 13

| | SPECIMEN* | | | CHANGE IN SPECIMEN | | | |
|---|---|---|---|---|---|---|---|
| | AGE | WT. | LENGTH | WEIGHT | | LENGTH | |
| STORING CONDITION | (DAYS) | (GM.) | (IN.) | GM. | % | IN. | % |
| Air, 63% relative humidity 25° C. | 0 | 95.210 | 2.5299 | 0.000 | 0.00 | 0.0000 | 0.000 |
| Air, 63% relative humidity 25° C. | 4 | 89.020 | 2.5298 | -6.190 | -7.72 | -0.0001 | -0.005 |
| Air, 63% relative humidity 25° C. | 6 | 88.615 | 2.5297 | -6.595 | -8.22 | -0.0002 | -0.009 |
| Air, 63% relative humidity 25° C. | 8 | 88.605 | 2.5297 | -6.605 | -8.24 | -0.0002 | -0.009 |
| Air, 63% relative humidity 25° C. | 10 | 88.610 | 2.5296 | -6.600 | -8.23 | -0.0003 | -0.014 |
| Air, 63% relative humidity 25° C. | 11 | 88.615 | 2.5296 | -6.595 | -8.22 | -0.0003 | -0.014 |
| Air, 63% relative humidity 25° C. | 14 | 88.570 | 2.5295 | -6.640 | -8.27 | -0.0004 | -0.019 |
| Air, 63% relative humidity 25° C. | 21 | 88.575 | 2.5296 | -6.635 | -8.27 | -0.0003 | -0.014 |
| Air, 63% relative humidity 25° C. | 28 | 88.610 | 2.5296 | -6.600 | -8.23 | -0.0003 | -0.014 |
| Water, 25° C. | 32 | 96.350 | 2.5298 | 1.140 | 1.42 | -0.0001 | -0.005 |
| Water, 25° C. | 36 | 97.005 | 2.5300 | 1.795 | 2.24 | 0.001 | 0.005 |
| Air, 63% relative humidity 25° C. | 42 | 88.320 | 2.5299 | -6.890 | -8.59 | 0.0000 | 0.000 |
| Air, 63% relative humidity 25° C. | 47 | 88.310 | 2.5298 | -6.900 | -8.60 | -0.0001 | -0.005 |
| Water, 25° C. | 49 | 95.610 | 2.5301 | 0.400 | 0.50 | 0.0002 | 0.009 |
| Water, 25° C. | 54 | 96.465 | 2.5302 | 1.255 | 1.56 | 0.0003 | 0.014 |
| Air, 63% relative humidity 25° C. | 56 | 88.030 | 2.5300 | -7.180 | -8.95 | 0.0001 | 0.005 |
| Air, 63% relative humidity 25° C. | 69 | 88.015 | 2.5299 | -7.195 | -8.96 | 0.0000 | 0.000 |
| Water, 25° C. | 84 | 97.160 | 2.5302 | 1.950 | 2.43 | 0.0003 | 0.014 |

*Gross weight of specimen ($W_o$) 95.210 Gm. Gross length of specimen ($L_o$) 2.5299 inches.
Weight of spheres 14.970 Gm. One-half diameter of spheres 0.4062 inch.
Net weight of specimen ($W_s$) 80.240 Gm. Net length of specimen ($L_s$) 2.1237 inches.

The specimen was allowed to remain in the mold until setting was completed. After removal from the mold, it was placed in a saturated atmosphere for one-half hour to allow it to cool to room temperature. This was necessary because the specimen was heated by the exothermic heat of the setting reaction. All subsequent readings were also made at room temperature (approximately 23° C.) to obviate thermal changes during measurement. The specimen was weighed to the nearest 5 mg. and this value recorded as $W_o$ (Table I). The length of the specimen was measured immediately by the method outlined above and recorded as $L_o$.

After these measurements had been made the specimen was stored in a controlled atmosphere approximating summer laboratory conditions (63 per

cent relative humidity, 25° C.). At various intervals the specimen was removed and again weighed and measured. After 4 weeks' storage under these conditions, it was immersed in water (25° C.). This wetting and drying cycle was repeated 3 times. The method used for measuring wet specimens was the same as that for dry ones except that the specimen was removed from the water, wiped with a towel, and weighed immediately.

The first 4 columns of Table I are relatively self-explanatory. They are all known or measured values. The last 4 columns are calculated values derived from the first 4. The changes in weight and length were found by subtracting the appropriate original value ($L_o$, $W_o$) from the value at any given time. This gives the total change in grams and inches. In order to compare specimens of slightly different weight and length, these changes were reduced to a percentage basis. To do this, a standard weight equal to the original weight minus the weight of the balls was used as the divisor. Similarly, a standard length equal to the original length minus the radius of each size ball was used to convert to per cent change in length. This length is the distance between the centers of the balls, and is the effective length of the specimen.

TABLE II

CHANGE IN DIMENSIONS OF DENTAL STONE AND PLASTER WITH LOSS IN WEIGHT CAUSED BY DRYING

| R & R WHITE CASTONE | | | | R & R MODEL PLASTER | | | |
|---|---|---|---|---|---|---|---|
| AGE (DAYS) | CONDITIONS OF TEST | % WEIGHT LOSS | CONTRACTION* (%) | AGE (DAYS) | CONDITIONS OF TEST | % WEIGHT LOSS | CONTRACTION* (%) |
| 1 | 63% relative humidity, 26° C. | 6.41 | 0.000 | 1 | 63% relative humidity, 25° C. | 6.70 | 0.004 |
| 4 | | 7.90 | 0.019 | 3 | | 18.2 | 0.000 |
| 5 | | 8.51 | 0.019 | 10 | | 20.8 | 0.016 |
| 9 | | 8.52 | 0.024 | 18 | | 20.8 | 0.021 |
| 18 | | 8.48 | 0.021 | 22 | | 20.8 | 0.021 |
| 25 | | 8.52 | 0.019 | 23 | Electric oven, 90° C. | 31.5 | 0.324 |
| 33 | | 8.49 | 0.019 | 24 | | 34.2 | 0.354 |
| 36 | | 8.51 | 0.019 | 25 | | 36.4 | 0.378 |
| 37 | Electric oven, 90° C. | 13.6 | 0.064 | 27 | | 36.5 | 0.378 |
| 38 | | 20.5 | 0.131 | 28 | | 36.4 | 0.386 |
| 39 | | 22.9 | 0.140 | 29 | | 36.6 | 0.382 |
| 40 | | 25.6 | 0.138 | 31 | | 36.7 | 0.400 |
| 42 | | 25.1 | 0.138 | 42 | | 36.7 | 0.410 |
| 43 | | 26.0 | 0.148 | 44 | Electric oven, 110° C. | 36.8 | 0.452 |
| 44 | | 26.2 | 0.153 | 46 | | 36.9 | 0.455 |
| 46 | | 26.2 | 0.162 | 48 | | 36.9 | 0.464 |
| 57 | | 26.4 | 0.180 | | | | |
| 59 | Electric oven, 110° C. | 26.4 | 0.223 | | | | |
| 61 | | 26.4 | 0.228 | | | | |
| 63 | | 26.4 | 0.237 | | | | |

*The contraction measurements are considered good to 0.004 per cent.

Table II is a summary of 4 data sheets such as Table I. It presents the average values for 2 dental stones and for 2 model plaster specimens undergoing a drying treatment. Each pair of specimens is a duplicate which was made the same day and received the same treatment, but each specimen represents a separate mix. The plaster specimens were made with a water powder ratio of 50:100, the stone specimens with a ratio of 30:100.

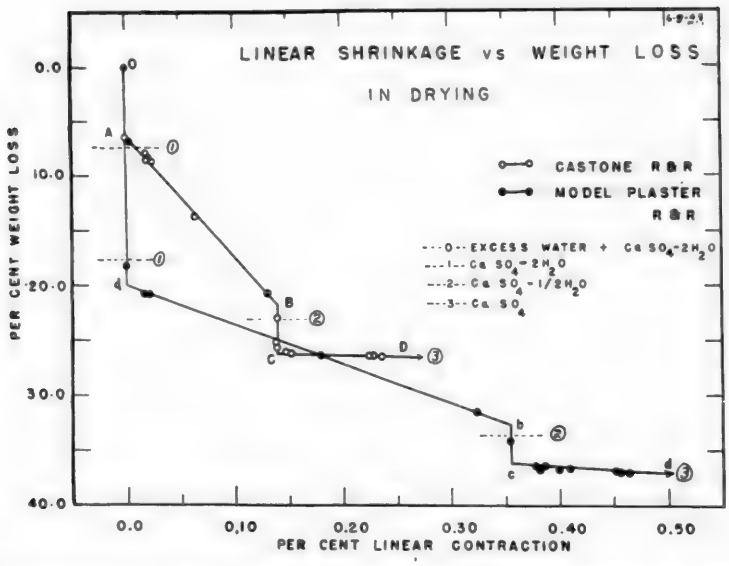


Fig. 3.—Comparison of weight loss and linear contraction of dental stone and model plaster.

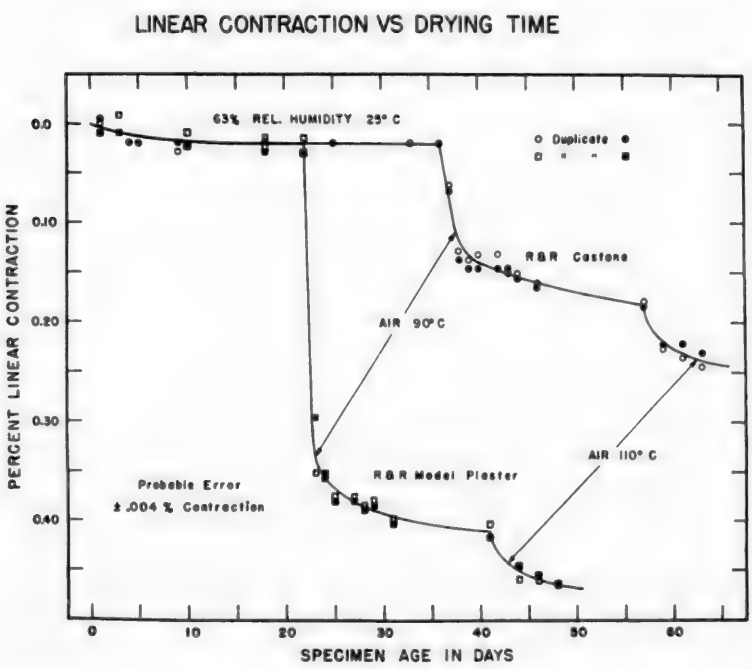


Fig. 4.—Dimensional change in dental stone and model plaster stored at 25° C., 90° C., and 110° C.

## RESULTS

In Fig. 3 is plotted the per cent weight loss and per cent contraction columns of Table II. It demonstrates the relationship between water loss in drying and the resulting shrinkage, and indicates that it is apparently closely related to the chemistry of the $CaSO_4$.

Knowing the water powder ratio used and assuming that the original powder was composed entirely of $CaSO_4 \cdot \frac{1}{2}H_2O$ it is possible to compute the weight loss required to remove all excess water and leave $CaSO_4 \cdot 2H_2O$. This weight loss is indicated as *1* on Fig. 3, and it is apparent that the removal of excess water has no appreciable effect on specimen length. Removal of more water will eventually transform the specimen from $CaSO_4 \cdot 2H_2O$ to $CaSO_4 \cdot \frac{1}{2}H_2O$, and the weight loss required to complete this transformation is indicated as *2*. It can be seen that this change is accompanied by a marked change in length. Removal of still more water results in a further rapid shrinkage, as $CaSO_4 \cdot \frac{1}{2}H_2O$ is changed to anhydrous $CaSO_4$, indicated as *3*.

This is of value to the dentist only if he can apply it to the selection of conditions that will prevent undesired dimensional change. In Figure 4 is presented, therefore, a graph of per cent contraction versus drying time for the same specimens, with the storage conditions as a parameter.

From this graph (Fig. 4) it appears that no significant change in dimension occurs as a result of storage of specimens under normal laboratory conditions, but that an increase in storage temperature leads to water loss and shrinkage. The calculated probable error of measurement of the data in Table II is ± .004 per cent contraction, and ± .006 per cent weight loss. This means that the measurement errors in each observation fall within the area of the dots used in Figs. 3 and 4, to represent an individual observation.

It should be emphasized that these data are included only as a demonstration that the suggested method of measurement has sufficient sensitivity to measure the dimensional changes that occur in gypsum products under the conditions encountered in dental practice. Until the authors' investigation of the storage properties of gypsum products is completed, they wish to draw no conclusions about them on the basis of the above data alone.

## CONCLUSIONS

The method described provides a means of determining the dimensional changes occurring in gypsum products with great sensitivity. It allows the use of a greater variety of treatments and avoids the failings inherent in previous methods used for this purpose.

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# THE EFFECT OF ZINC CHLORIDE AND POTASSIUM FERROCYANIDE AS A CARIES PROPHYLAXIS

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CLAIMS have been made that zinc chloride and potassium ferrocyanide are effective in reducing the incidence of dental caries[1] but the supporting evidence has not been convincing[2, 3] or substantiated by laboratory studies[4] or other workers.

In an effort to test the hypothesis under controlled conditions, 100 Laramie, Wyoming, school children were examined during December, 1948. The children selected gave histories of continuous residence in Laramie and only those whose parents gave consent were used in the study. An interested group of Laramie dentists in Albany County, Wyoming, known as the Research Committee, agreed to apply the drugs to the children who had been examined.

## METHOD

The dental examinations of the children were performed by the author in the nurse's office of the schools, using a portable and an adjustable dental chair, natural light supplemented by a portable focusing artificial light, and using new mouth mirrors and sharp explorers. The findings were recorded on a form, according to the method recommended by Knutson.[5] Names of the children so examined were given to the Research Committee who treated the children in the manner advocated by Gottlieb.[6]

Following the original examination, the children were brought to the multi-chair office of one of the dentists in groups of 10 or 15 on specific afternoons set aside for this purpose. Except for a few who were treated in December, 1948, the entire group of more than 100 children received the treatment in one-half of the upper teeth and one-half of the lower teeth during January, 1949. The quadrants chosen to receive the treatment were changed on different days so that, roughly, one-half the children had the upper left and lower right quadrants treated and the remaining teeth used for controls while the other half of the children had the upper right and lower left quadrants treated and their opposites used for controls.

At the time the children were brought to the dental office for the prophylatic treatment they were again examined by the local dentists and bilateral posterior bitewing roentgenograms were taken for a record.

The age range of the children was from 8 to 14 years, inclusive, at the close of the study. The following technic was meticulously used: (1) A prophylaxis was given with flour of pumice and a rubber cup. Pumice was carried through the interproximal areas with dental floss; (2) a rubber dam was placed to expose all the teeth in the treated quadrant; (3) the field was dried with warm air;

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(4) the teeth were washed for one minute or longer with hydrogen peroxide to which one or two drops of Nacconol* had been added; (5) the field was again dried with warm air; (6) the teeth were then washed with alcohol for one minute and dried with warm air; (7) all tooth surfaces were moistened with Nacconol and partially dried with air; (8) all tooth surfaces were then painted with a cotton pellet held in cotton pliers and saturated with Nacconol and zinc chloride (50 per cent solution); this solution was permitted to remain on the teeth for two minutes; (9) the tooth surfaces were painted once more with a cotton pellet saturated with Nacconol and potassium ferrocyanide (20 per cent solution); the solution was rubbed on the teeth until a heavy milky precipitate was formed; (10) the teeth were finally dried and the rubber dam removed; and (11) the opposite teeth in the opposite jaw were then isolated and the foregoing treatment applied.

In March, 1950, the school nurse supplied the author with a list of about 100 children who had received the single application of drugs the previous year. The re-examinations were made under the same physical conditions as originally and the data recorded on new forms in the same manner as the original examinations. At the second examination there was no record available to the examiner as to which teeth had been treated and it was not obvious that one quadrant was any "cleaner" or had more plaques than the other.

After charting the mouths the second time, the examination cards were taken to the dental office where the treatment records were made available. The treated quadrants were marked on the second examination form and the analysis made accordingly. Following this, the Research Committee re-examined the children and took follow-up bitewing roentgenograms. Their findings may be the subject of another report and are not included here.

During the study year, a few children had moved from Laramie and a few were absent at the time of the second examination. Several children were treated who had not been examined originally by the author with the result that only 95 children are included in this analysis.

## FINDINGS

Table I shows the data for the treated and untreated deciduous teeth that were present at the time of both examinations. Caries experience is expressed in terms of the number of teeth and tooth surfaces initially attacked during the study period and addtional tooth surfaces attacked in teeth that were carious in December, 1948, and present in the mouth at the time of the second examination.

According to the data presented in Table I, a total of 10 sound deciduous teeth became carious in the treated quadrants as compared with 5 deciduous teeth in the untreated quadrants. In other words, 9.9 per cent of the noncarious deciduous teeth in the treated quadrants became carious during the study period as compared to 5.3 per cent in the untreated quadrants. While the number of teeth that became carious in both the treated and untreated quadrants is small, a conservative observation is that these data reflect no benefit to deciduous teeth by using zinc chloride and potassium ferrocyanide.

*National Aniline & Chemical Co. Inc., New York, N. Y.

TABLE I

DENTAL CARIES EXPERIENCE DURING 13-MONTH* PERIOD ENDING MARCH, 1950, FOR DECIDUOUS TEETH IN TREATED (ZINC CHLORIDE AND POTASSIUM FERROCYANIDE) AND UNTREATED QUADRANTS OF MOUTHS OF 95 LARAMIE, WYOMING, SCHOOL CHILDREN, AGES 8 THROUGH 14 YEARS

| QUADRANT | NUMBER OF NONCARIOUS TEETH (DEC., 1948) | NEW DF† TEETH (MAR., 1950) | DF SURFACES IN NEW DF TEETH | NEW DF SURFACES IN PREVIOUSLY CARIOUS TEETH | TOTAL NEW DF SURFACES |
|---|---|---|---|---|---|
| | | *Upper* | | | |
| Treated | 65 | 8 | 11 | 26 | 37 |
| Untreated | 54 | 4 | 4 | 23 | 27 |
| | | *Lower* | | | |
| Treated | 36 | 2 | 2 | 22 | 24 |
| Untreated | 40 | 1 | 1 | 14 | 15 |
| | | *Both Jaws*‡ | | | |
| Treated | 101 | 10 | 13 | 48 | 61 |
| Untreated | 94 | 5 | 5 | 37 | 42 |

*Treatment carried out during December, 1948, and January, 1949.
†DF = carious (decayed or filled).
‡Deciduous teeth present at time of both examinations only included.

In Table II, 72 permanent teeth in the treated quadrants became carious during the study period campared to 71 permanent teeth in the untreated quadrants. The per cent of teeth becoming carious were 12.7 and 12.3, respectively. A comparison of the number of permanent tooth surfaces attacked by caries is essentially the same, although the slight difference (0.9 per cent) favors the treated quadrants.

TABLE II

DENTAL CARIES EXPERIENCE DURING THIRTEEN-MONTH* PERIOD ENDING MARCH, 1950, FOR PERMANENT TEETH IN TREATED (ZINC CHLORIDE AND POTASSIUM FERROCYANIDE) AND UNTREATED QUADRANTS OF MOUTHS OF 95 LARAMIE, WYOMING, SCHOOL CHILDREN, AGES 8 THROUGH 14 YEARS

| QUADRANT | NUMBER OF NONCARIOUS TEETH (DEC., 1948) | NEW DF† TEETH (MAR., 1950) | DF SURFACES IN NEW DF TEETH | NEW DF SURFACES IN PREVIOUSLY CARIOUS TEETH | TOTAL NEW DF SURFACES |
|---|---|---|---|---|---|
| | | *Upper* | | | |
| Treated | 256 | 40 | 46 | 28 | 74 |
| Untreated | 273 | 44 | 55 | 30 | 85 |
| | | *Lower* | | | |
| Treated | 310 | 32 | 37 | 21 | 58 |
| Untreated | 300 | 27 | 34 | 30 | 64 |
| | | *Both Jaws* | | | |
| Treated | 566 | 72 | 83 | 49 | 132 |
| Untreated | 573 | 71 | 89 | 60 | 149 |

*Treatment carried out during December, 1948, and January, 1949.
†DF = carious (decayed or filled).

There were also slightly fewer (132) total number of carious surfaces occurring in the permanent teeth of the treated quadrants when compared with the untreated quadrants (149). In the light of the finding that the number of teeth becoming carious during the study year was essentially the same in the treated and untreated quadrants, the slight variation in the number of surfaces involved in newly carious (during study period) and previously carious (newly decayed and filled surfaces in previously carious) teeth seems to have little significance.

In short, these data indicate that one application of zinc chloride and potassium ferrocyanide does not inhibit dental caries incidence.

## SUMMARY

The conditions of this study (treatment of a fairly large number of children; examinations before and after treatment by the same dentist; no knowledge of the treated quadrants at the time of the concluding dental examinations; and applications of the drugs by a competent group of dentists familiar with the technic) indicate that zinc chloride and potassium ferrocyanide are not effective in controlling the incidence of new caries. The claims for the prophylactic effect of the so-called impregnation technic are, therefore, not supported.

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# VITAMIN REQUIREMENTS OF ORAL LACTOBACILLI

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IN CONSIDERING the relationship of oral bacteria to conditions arising in the mouth, the advantage of a thorough knowledge of metabolism of the microorganisms often has been apparent. One important phase of microbic metabolism is the vitamin requirement. It is now well established that certain bacteria are unable to synthesize some of the vitamins needed in their metabolic processes. This is important from the standpoint of development of the organism, for if a needed vitamin or a satisfactory substitute (of which there are few known) is lacking, cell metabolism and multiplication stop even when other environmental conditions are appropriate. When the vitamin is supplied these processes are carried on readily. Thus it happens that growth of such bacteria is dependent upon a supply of the preformed vitamin, and also that the extent of growth and of resultant metabolic products, such as acid produced from sugars, often is proportional within certain limits to the concentration of the vitamin.

Since the oral lactobacilli are associated prominently with the carious process, it was deemed desirable to know more of their nutritive requirements and particularly of the vitamin requirement. Previous studies of other workers have given some insight into this problem. Hill and Kniesner[1] reported that 9 strains of oral lactobacilli all needed pantothenic acid and that the amount of panthothenic acid in human saliva is usually sufficient for optimal growth of these organisms. Weisberger and Johnson[2] found that thiamine, pantothenic acid, nictotinic acid, and tryptophane were needed by an oral lactobacillus. When these substances were supplied the organism grew readily and produced maximal acid in a hydrolyzed casein digest-glucose-salts medium. Riboflavin, biotin, pyridoxine, folic acid, and some purines and pyrimidines were nonessential for this organism. Coolidge, Williams, Ebisch, and Hodges[3] noted that nicotinic acid, pantothenic acid, biotin, thiamine, and riboflavin were important in the nutrition of several strains of oral lactobacilli which were used in a study of variation in fermentation and other metabolic properties. The current study of classification of lactobacilli reported by Rogosa, Mitchell, and Fitzgerald[4] promises to supply much needed information. These workers used many oral strains and included the vitamin needs of the organisms along with other characteristics used for separating different types or species. From their preliminary report it is evident that pantothenic acid, nicotinic acid, biotin, folic acid, thiamine, riboflavin, vitamin $B_6$, and vitamin $B_{12}$

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are important in the nutrition of these organisms. Certain relationships between the need for some of these vitamins and other characteristics were brought out.

These reports give us some information of the vitamin requirements of the oral lactobacilli. But it is also evident that up to the present time our knowledge of the needs of these organisms has not kept pace with that of some other bacterial groups. Among the closely related types, the vitamin needs of lactobacilli from sources other than the mouth, of some of the lactic acid streptococci, and of the related Leuconostoc have been studied intensively.[5] In the present work we have studied the vitamin needs of a series of oral lactobacilli but made no attempt at this time to identify the different strains used in this study with the species names now used in literature. Since there is at present much confusion in the separation of types or species in this group of bacteria it was felt that a better knowledge of nutritional needs might assist in any later classification of the group, and that such separation might be deferred until more information has become available.

## METHODS

*Cultures.*—Twenty-six strains of oral lactobacilli from human sources were included in the study. They were isolated from saliva of caries-active patients, from plaques, and from cavities. Eleven of the 26 were stock cultures which had been maintained in the laboratory collection for a number of years. The remaining 15 cultures were more recently isolated,* some of them being subjected to only the few transfers on laboratory culture media necessary for their isolation before the tests of vitamin requirements were started.

*Basal Medium.*—To study the effect of added vitamins it is necessary to employ a basal medium which contains all needed amino acids in adequate amounts, a readily available source of energy, inorganic salts, and which is suitable in other respects except for its deficiency in vitamins. Since the exact amino acid needs of the oral lactobacilli are not known, an acid hydrolyzate of casein was used to supply different amino acids. This was supplemented with several amino acids which are contained in casein in only small amounts or which might be destroyed in the process of acid hydrolysis. The detailed composition per 1,000 ml. of medium follows: 5.0 Gm. "vitamin-free" hydrolyzed casein, 100 mg. L-cystine, 100 mg. L-tryptophane, 100 mg. L-hydroxyproline, 200 mg. DL-threonine, 200 mg. DL-serine, 1.0 Gm. $KH_2PO_4$, 1.0 Gm. $K_2HPO_4$, 10 mg. NaCl, 200 mg. $MgSO_4 \cdot 7H_2O$, 10 mg. $FeSO_4 \cdot 7H_2O$, 7.5 mg. $MnCl_2 \cdot 4H_2O$, 6.0 Gm. sodium acetate, and 10.0 Gm. glucose. The compounds are dissolved in 800 to 900 ml. of glass redistilled water and the volume made up to 1,000 ml. later.

The cystine was dissolved in a small amount of N/1 HCl (usually 2 ml. of the acid per 100 mg. cystine) and then added to the medium; the other compounds were added directly, warming to facilitate solution. The medium was adjusted to pH 6.8 using NaOH pellets. This increased the amount of sodium

*We are indebted to members of the clinics staff of the Walter G. Zoller Memorial Dental Clinic for collection of the specimens from which these isolations were made.

in the medium to 0.3 to 0.5 Gm. per liter. The medium was next filtered to remove the light haziness or turbidity which appears. This is evidently due largely to precipitated salts of iron and manganese. The amounts of iron and manganese remaining in solution are not known, but apparently are sufficient for luxuriant growth of the organisms in the presence of other required nutritive substances. On omission of the iron and manganese the light precipitate was avoided, but comparative growth tests showed this omission resulted in a slower and poorer growth response on the part of many cultures.

The completed medium was dispensed in 10 ml. amounts in 18 by 150 mm. test tubes previously standardized for use in the Lumetron colorimeter, and then sterilized in the autoclave at 10 pounds extra pressure for 15 to 20 minutes. After autoclave sterilization the pH was found to be between 6.6 and 6.7.

Occasional slight departures were made on some occasions. When a number of different vitamin solutions were to be added to tubes of the basal medium, thereby increasing the volume somewhat, the ingredients for 1000 ml. of medium were dissolved in a total of 800 or 900 ml. of redistilled water and tubed in 8.0 or 9.0 ml. amounts instead of 10 ml. If subsequent addition of the different accessory factor solutions did not bring the total volume of each tube to approximately 10 ml., sufficient sterile redistilled water was added for this purpose. Certain other experimental changes in the composition of the basal medium, which were made for the purpose of determining the particular combination of ingredients for best growth of the lactobacilli, will be mentioned as the results are presented.

*Vitamin and Other Growth Factor Solutions.*—Stock solutions of these compounds were made in redistilled water in concentrations of 100 or 1,000 micrograms ($\mu$g.) per ml. or occasionally larger amounts. Most of them were sterilized by filtration through fritted glass filters, but a few known to be stable to heat were sterilized in the autoclave. From the stock solutions known amounts were added aseptically to tubes of the previously sterilized basal medium. The different combinations and the amounts used are given later with the results.

*Inoculation.*—The inoculum transferred to each tube in the tests for growth consisted usually of 0.001 ml. of a 22- to 24-hour culture grown at 37° C. in tomato juice yeast extract broth. This amount of culture was obtained by dilution in phosphate-buffered physiological salt solution. The small quantity of vitamins or other factors carried over with the cells in an inoculum of this size was found to be insufficient to support any real growth of lactobacilli in the unsupplemented basal medium.

*Measurement of Growth.*—All cultures were incubated at 37° C. The growth response to the various vitamin supplements was measured by turbidity with a Lumetron colorimeter, using a 650 m$\mu$. filter with an uninoculated tube of medium set at 100 as the blank. In the following tables of data, such a clear tube with full light transmission is designated as 0, or no turbidity. Increasing turbidities due to growth are shown by progressively higher figures. A figure of 60 to 80 represents heavy turbidity. When stains were made directly from such cultures they showed the presence of many lactobacilli in each micro-

scope field. Readings of growth were made usually at 24-hour intervals for the first 3 or 4 days after inoculation, then once or twice more with the final reading at 7 days or occasionally later.

## RESULTS

*Growth Response to a Mixture of Known Vitamins.*—The first experiments were designed to determine whether the organisms were capable of growing when a mixture of known vitamins was added to the casein digest basal medium. The results with about half of the cultures, which are representative of others, are given in Table I. In the unsupplemented basal medium all oral lactobacilli failed to grow. The very slight turbidity represented by the numerals 1, 2, etc., results probably from traces of impurities in the ingredients of the medium and from the carry over of minute amounts of vitamins in the cells used for inoculation. In contrast to these results are those in the same medium plus the vitamin supplement. Here cell metabolism and multiplication have progressed to the point where a marked turbidity of the culture tube is readily apparent, though often several days are required for the organisms to attain a colorimeter reading within the range of 50 to 80, which is roughly equivalent to quite good or luxuriant growth. The difference between these cultures, which developed in the presence of the vitamins, and the control tubes is evident by ordinary inspection, but the colorimeter figures serve to show more exactly the differing rates of development of the various cultures. In a few instances, such as numbers 41, 1001, and 1008 of Table I, quite good growth appeared within 24 hours. With about one-half of the cultures, maximal or near maximal growth was attained in 48 hours in the presence of the vitamin supplement. Some cultures were appreciably slower in their development, for example, numbers 4, 26, 1005, and especially 1002. It would seem, in these cases, factors other than those supplied in the experimental medium are needed for prompt growth. However, the need for such substances is evidently not an absolute one since slow growth appears without them. Evidently the bacteria in these cases are able to synthesize slowly other needed growth factors, or else the basal medium or conditions of cultivation are not best suited to these particular strains. Eventually, however, the cultures produced quite good growth, though at times incubation for 7 days was required.

*Cell Counts.*—To gain an idea of the numbers of cells after growth in casein hydrolyzate medium supplemented with vitamins, plate counts were made of some representative strains. Tomato juice yeast extract agar, pH 6.5, was used as the plating medium. It was found that Cultures 3, 4, 19, and 96 attained counts of 255 million to 900 million cells per ml. in the vitamin-supplemented casein digest medium after 48 or 72 hours at 37° C. The colorimeter readings of these cultures at the time samples were withdrawn for plating ranged from 70 to 84.

Plate counts also were made to determine the number of cells in the inoculation which the tubes in the vitamin tests received. The 0.001 ml. of 22- to 24-hour culture used for inoculation yielded counts, for the most part, between 340,000 and 700,000. Since the tubes contained 10 ml. of the test me-

dium, this resulted in an inoculation of 34,000 to 70,000 cells per ml. of medium. Such numbers, of course, are much lower than those obtained by direct transfer of a loopful of liquid culture, but are sufficiently large to permit ready development of the culture if the organism is able to make use promptly of the materials provided.

*Effect of Changes in the Basal Medium.*—From the foregoing results as shown in Table I, it is apparent that the oral lactobacilli are capable of growing in the basal medium when a supplement of B vitamins is added, and also that the rate of growth often is appreciably slower than that seen in culture media made from natural products such as yeast extract and tomato juice.

TABLE I

EFFECT OF A MIXTURE OF KNOWN VITAMINS ON GROWTH OF SOME ORAL LACTOBACILLI IN A VITAMIN-DEFICIENT BASAL MEDIUM

| CULTURE NUMBER | BASAL MEDIUM TURBIDITY AT 7 DAYS | BASAL MEDIUM PLUS MIXTURE OF VITAMINS* TURBIDITY AT DAYS | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 7 |
| 3 | 1 | 3 | 56 | 76 | | |
| 4 | 1 | 1 | 5 | 45 | 78 | |
| 9 | 0† | 2 | 20 | 68 | 71 | |
| 19 | 1 | 15 | 66 | 71 | 75 | |
| 26 | 2 | 1 | 6 | 42 | 80 | |
| 41 | 1 | 54 | 76 | 76 | | |
| 641 | 0 | 3 | 37 | 86 | | |
| 1001 | 0 | 67 | 85 | | | |
| 1002 | 0 | 0 | 0 | 0 | 3 | 75 |
| 1003 | 1 | 15 | 84 | | | |
| 1005 | 0 | 0 | 2 | 38 | 72 | |
| 1008 | 1 | 54 | 71 | 75 | | |

*The vitamins used and the final amounts per ml. of medium were: nicotinic acid, thiamine hydrochloride, calcium pantothenate, pyridoxamine hydrochloride, each 1.0 μg; riboflavin 0.2 μg; pteroylglutamic acid and para-aminobenzoic acid each 0.1 μg; biotin 0.01 μg. Most of the vitamin solutions were sterilized by filtration and added to the previously autoclaved tubes of medium.

†0 indicates a clear tube, similar to the uninoculated medium; higher figures represent increasing turbidities due to growth of the cultures. Values from about 60 to 80 are very turbid cultures, i.e., heavy growth by ordinary visual inspection. Later readings are not given after cultures attained full growth or practically full growth.

While this slower growth may well be due to a deficiency of still other substances, such as purines, pyrimidines, fat-soluble compounds, or others, there is the possibility that certain changes in the composition of the basal medium itself might lead to a more favorable growth response. It seemed important to determine the optimum composition of the basal medium before starting tests of the vitamin requirements of individual cultures. Accordingly the effect of a number of alterations in the basal medium was tested, particularly with respect to acetate, addition of citrate, nitrogen in the form of an ammonium salt, and the amounts of the inorganic salts. Not all of the lactobacilli were used for each test of the various combinations of ingredients, but 10 to 12 strains were selected as representative from both the rapid and the slow growing types. Their growth response in each modification of the basal medium was compared to that in the standard basal medium which has been given in a previous section of this paper. The same vitamins were included in equal amounts in each case. The principal changes in the basal medium which were subjected to test are summarized in the following outline:

A. The addition of 0.5 per cent NaCl, or 0.3 per cent $(NH_4)_2SO_4$, or both.

B. Omission of the 0.6 per cent sodium acetate and substitution of 0.3 per cent sodium citrate.

C. Inclusion of the usual 0.6 per cent sodium acetate with the addition of 0.3 per cent sodium citrate and 0.3 per cent $(NH_4)_2SO_4$.

D. Substitution of 0.6 per cent ammonium acetate for the usual sodium acetate, with addition of 0.5 per cent sodium citrate.

E. Reduction in the amounts of iron and manganese salts, or omission of them.

F. Addition of 0.1 per cent $NaHCO_3$.

G. Addition of a reducing agent, 0.1 per cent sodium thioglycollate.

H. An increase in the amount of casein hydrolyzate and the added amino acids to twice that usually used.

The supporting data for these numerous experiments are too voluminous to be presented here in detail but the results of comparative growth tests can be summarized briefly. The addition of NaCl and an inorganic ammonium salt (A) did not produce appreciably speedier growth in so far as the average result of all cultures was concerned; while a few were benefited to some extent, growth of others was slightly retarded. Substitution of citrate for acetate (B) resulted in appreciably speedier growth of about one-half of the cultures, but one strain was definitely retarded. The addition of 0.5 per cent NaCl, or 0.3 per cent $(NH_4)_2SO_4$, or both, was also tried along with the sodium citrate with 7 cultures but gave no better over-all results; here again, an occasional slight retardation of growth tended to balance the occasional beneficial effect. When citrate and $(NH_4)_2SO_4$ were added to the basal medium (C) 4 of the cultures exhibited speedier growth, with 2 of the 4 being quite definitely better; 6 cultures showed no real differences in the rate of growth, while 2 cultures were very definitely retarded. With combination D the results were rather similar to those of C: 5 cultures showed accelerated growth though in several instances this effect was not marked, 6 cultures were about the same, and 2 were retarded in the rate of growth.

The omission of iron and manganese from the basal medium (E) produced a very definite retardation of growth and in some instances inhibited it completely. Reduction in the amounts of these 2 metallic ions to one-fifth of that usually used caused distinct delay in initiation of growth. The incorporation in the medium of sodium bicarbonate (F) as a source of $CO_2$ did not cause any over-all improvement; growth of a few strains was slightly accelerated, others were retarded. Similarly, the addition of a reducing agent (G) produced no general improvement in either the rate or final luxuriance of growth. Doubling the amount of amino acids and products of protein hydrolysis (H) produced a slightly accelerated growth in some instances as well as a little more luxuriant final growth. This increased amount of nitrogenous foodstuff was not adopted for regular use, however, because it produced a doubling in the amount of any vitamins or growth factors which might be present as impurities in the casein hydrolyzate. This in turn would cause growth tests with the individual vitamins to be less clear cut.

Since the various alterations in the basal medium produced no really definite beneficial effect that applied to all cultures, it was decided to continue with the basal medium as given in the original outline. The incorporation of citrate would have seemed desirable, since growth of a number of cultures was stimulated, had it not been for the distinctly retarding effect exerted in the case of 2 of the cultures.

*Vitamins Required.*—The next step was to determine the more exact needs of each culture by omitting the different vitamins one at a time from the mixture. It was soon found that the omission of para-aminobenzoic acid had little or no effect upon the rate or luxuriance of growth. Evidently this compound is not needed in the hydrolyzed casein medium. Whether a need for it might be demonstrated by using a specially purified casein was not determined. This is a possibility however, for in experiments with *Lactobacillus pentosus* Krueger and Peterson[6] noted some evidence of a para-aminobenzoic requirement when a specially prepared casein was used. The ordinary "vitamin-free" casein may contain traces of para-aminobenzoic acid or something which substitutes for it physiologically.

The results of some experiments on omission of single vitamins are given in Table II. This shows 4 cultures which are representative of most of the others. The results are given for only one time interval, a 3-day incubation period, which is sufficient for comparison of the cultures. In the actual tests, however, turbidity readings were made daily for the first 4 days and again at 7 days, as shown in Table I. It is apparent that the vitamin requirement is rather complex, since each organism needs 5 or 6 of the vitamins for prompt, vigorous growth.

TABLE II

EFFECT ON GROWTH OF OMISSION OF SINGLE VITAMINS FROM MIXTURE

| VITAMINS | GROWTH AT 3 DAYS* LACTOBACILLUS NUMBER | | | |
|---|---|---|---|---|
| | 41 | 45 | 96 | 1009 |
| 1. Basal medium | 1 | 0 | 1 | 1 |
| 2. Basal medium plus 7 vitamins† | 76 | 75 | 81 | 69 |
| 3. No. 2 minus nicotinic acid | 1 | 15 | 9 | 8 |
| 4. No. 2 minus pantothenic acid | 1 | 3 | 7 | 5 |
| 5. No. 2 minus biotin | 9 | 7 | 12 | 6 |
| 6. No. 2 minus pyridoxamine | 64(+) | 31(±) | 17‡(±) | 63 |
| 7. No. 2 minus pteroylglutamic acid | 26(±) | 3(−) | 7(−) | 2 |
| 8. No. 2 minus riboflavin | 75(+) | 2 | 3 | 1 |
| 9. No. 2 minus thiamine | 2 | 75(+) | 80(+) | 75 |

*The figures represent the amount of growth and are given on the same basis as those in Table I.

The results of successive transfers are indicated by the symbols in parentheses following the turbidity reading:

(+) Grew readily through 10 successive transfers.

(±) Grew slowly or poorly, but could be carried through 10 successive transfers.

(−) Failed to grow in successive transfers.

†The 7 vitamins and the amounts used are the same as given in Table I, except that para-aminobenzoic acid is omitted here.

‡Heavier growth, 45 to 50, was secured on continued incubation up to 7 days.

The omission of either nicotinic acid, pantothenic acid, or biotin results in failure of growth. These 3 compounds are definitely needed. The low turbidity figures (3, 7, 8, 9, etc.) obtained in the absence of these compounds are

not significant with respect to real growth, but are the result of traces of these vitamins either in the basal medium or in the inoculum. With respect to the individual cultures, Number 41 grew quite well in the absence of pyridoxamine or riboflavin (compare lines 6 and 8 with line 2), but attained only mediocre development when pteroylglutamic acid was omitted. By the use of successive transfers in the pteroylglutamic acid-free medium it was found, however, that Culture 41 was capable of continuous growth through 10 such transfers, though growth was slow and rather light. Evidently pteroylglutamic acid is not an absolute need for this organism but its presence facilitates growth. Culture 41 needs thiamine, for on omission of this vitamin there is no growth.

In a similar way the vitamin needs of Cultures 45, 96, and 1009 were determined. In addition to nicotinic acid, pantothenic acid, and biotin, they require pteroylglutamic acid and riboflavin, but not thiamine. These 3 cultures differ slightly in their response to the vitamin $B_6$ compound, pyridoxamine. Number 45 grew only poorly in its absence, but nevertheless could be carried through 10 successive transfers when this compound was omitted. Culture 96 in the absence of pyridoxamine eventually attained moderately good growth after 7 days of incubation and could be carried through successive cultures if sufficient time were allowed between transfers. Culture 1009, unlike 45 and 96, grew promptly and vigorously in the absence of pyridoxamine.

In a similar way the vitamin needs of the other strains of oral lactobacilli were determined. The results on all cultures are summarized briefly in Table III. Nicotinic acid, pantothenic acid, and biotin were needed by all 26 cultures. Riboflavin was needed by many of the different strains, thiamine by a few. Need for pyridoxamine and for pteroylglutamic acid was not always as definitely shown as in the case of the other vitamins. While some strains exhibited a definite need for these compounds there were a number of others which grew to some extent without them, but whose growth was facilitated by the presence of one or the other or both of these substances (in general, similar to the results shown for Cultures 41, 45, and 96 in Table II).

The usual interpretation of results such as those seen in the pyridoxamine and pteroylglutamic acid columns in Table III is that some organisms are able to synthesize the vitamin promptly enough for speedy growth, others are partially deficient in synthesizing ability and are able at best to form the compound slowly, while still others are totally unable to make it and so are dependent on an extraneous source of the vitamin. While this explanation may well apply in these instances also, it is somewhat clouded by the possibility of substitution or partial substitution of other compounds for these vitamins. Thus, it is known that thymine in larger quantities may replace pteroylglutamic acid (folic acid) for some lactic acid bacteria.[7, 8] Thymine was not added to the medium in the tests shown in Table III, but other compounds also have been reported to show some folic acid activity. Hall[9] reported that histidine, when sterilized in the medium by autoclaving, replaced folic acid to some extent in the growth of *Streptococcus faecalis* R. and Holland and Meinke[10] noted an effect between serine and folic acid for the same organism. There is the possibility that such substitutions may have played some part in the results with pteroylglutamic acid.

An interrelationship between pyridoxamine, or other vitamin $B_6$ compounds, and certain amino acids is now well established.[11-14] In the presence of certain amino acids, such as lysine, threonine, alanine, and others, the vitamin $B_6$ requirement of several lactic acid bacteria is decreased or eliminated; in the absence of these amino acids an adequate supply of the vitamin is essential for growth. Since hydrolyzed casein was used for the basal medium it is evident that certain amino acids may have substituted to some extent for pyridoxamine in the case of some of the lactobacilli, but not necessarily for all of them. Additional experiments with synthetic basal medium prepared only with certain amino acids would be needed to determine more exactly the vitamin $B_6$ and pteroylglutamic acid (or folic acid) needs of these organisms.

TABLE III

SUMMARY OF VITAMIN NEEDS OF 26 STRAINS OF ORAL LACTOBACILLI

| CULTURE NUMBER | NICOTINIC ACID | PANTOTHENATE | BIOTIN | PYRIDOXAMINE | PTEROYLGLUTAMIC ACID | RIBOFLAVIN | THIAMINE |
|---|---|---|---|---|---|---|---|
| 3 | + | + | + | +* | + | + | – |
| 4 | + | + | + | + | + | + | – |
| 9 | + | + | + | + | + | + | – |
| 19 | + | + | + | +* | + | + | – |
| 26 | + | + | + | + | + | + | – |
| 41 | + | + | + | – | +* | – | + |
| 45 | + | + | + | +* | + | + | – |
| 46 | + | + | + | + | + | + | – |
| 78 | + | + | + | – | + | – | + |
| 96 | + | + | + | +* | + | + | – |
| 641 | + | + | + | + | + | + | – |
| 1001 | + | + | + | – | +* | – | – |
| 1002 | + | + | + | – | – | – | – |
| 1003 | + | + | + | + | + | + | – |
| 1004 | + | + | + | +* | – | – | + |
| 1005 | + | + | + | + | + | + | – |
| 1006 | + | + | + | +* | + | + | – |
| 1007 | + | + | + | – | – | – | + |
| 1008 | + | + | + | – | – | – | + |
| 1009 | + | + | + | – | + | + | – |
| 1010 | + | + | + | – | + | + | – |
| 1011 | + | + | + | + | + | + | – |
| 1012 | + | + | + | +* | + | + | – |
| 1013 | + | + | + | – | – | – | + |
| 1014 | + | + | + | + | + | + | – |
| 1015 | + | + | + | – | – | – | + |

+ Required for growth.
– Not required.
+* Needed for prompt and vigorous growth, but poorer or slower growth without the vitamin.

There is an interesting inverse relationship in the need for riboflavin and thiamine; strains needing one vitamin do not need the other. Rogosa, Tittsler, and Geib,[15] in a study of many different lactobacilli from various sources, noted that the heterofermentative types require thiamine, and they noted also the inverse relationship in the thiamine and riboflavin requirement.[16] The results with the oral lactobacilli shown in Table III are in harmony with their findings with the exception of one culture, number 1001. A rough test for the heterofermentative type of metabolism is gas production in a deep agar column in a

suitable growth medium with glucose. This test was applied to all of our cultures and found to correlate well with the thiamine requirement.

*Successive Transfers.*—Transfers in series in the different vitamin combinations were employed in many cases to check the results. Such successive transfers were applied particularly to the vitamin combinations determined to be needed on the basis of individual omissions and also as an aid in clarifying the pyridoxamine and pteroylglutamic acid results, as previously noted in the several cultures given in Table II. Tests with other cultures were made but are not given in detail although the successive transfer results were always taken into account in compiling the list of vitamin needs shown in Table III.

TABLE IV

ACCELERATION OF GROWTH BY (1) PURINES, PYRIMIDINES, AND GLUTAMINE, AND (2) YEAST EXTRACT

| | BASAL MEDIUM PLUS | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | VITAMINS* | | | | VITAMINS PLUS PURINES, PYRIMIDINES,† AND GLUTAMINE | | | YEAST EXTRACT 0.2% | | |
| | DAYS | | | | DAYS | | | DAYS | | |
| CULTURE | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 1 | 2 | 3 |
| 3 | 2 | 46 | 75 | | 7 | 65 | 73 | 50 | 65 | 70 |
| 4 | 1 | 4 | 35 | 73 | 57 | 79 | 81 | 81 | | |
| 9 | 2 | 39 | 80 | | 3 | 44 | 80 | 80 | | |
| 19 | 11 | 74 | 78 | | 11 | 71 | 81 | 77 | | |
| 26 | 1 | 6 | 42 | 71 | 5 | 68 | 78 | 77 | | |
| 41 | 4 | 83 | | | 76 | 84 | | 83 | | |
| 45 | 13 | 75 | 78 | | 57 | 86 | | 84 | | |
| 46 | 11 | 85 | | | 69 | 88 | | 82 | | |
| 78 | 2 | 82 | | | 3 | 85 | | 82 | | |
| 96 | 7 | 81 | | | 44 | 80 | | 80 | | |
| 641 | 1 | 16 | 81 | | 23 | 87 | | 82 | | |
| 1001 | 37 | 60 | 62 | | 80 | 80 | | 50 | 70 | |
| 1002 | 0 | 0 | 1 | 6‡ | 7 | 63 | 58 | 29 | 57 | 58 |
| 1003 | 0 | 35 | 68 | | 17 | 80 | | 74 | 84 | |
| 1004 | 54 | 54 | | | 65 | 65 | | 67 | 77 | |
| 1005 | 0 | 5 | 23 | 62 | 0 | 10 | 72 | 47 | 80 | |
| 1006 | 0 | 5 | 17 | 56 | 8 | 75 | | 57 | 71 | |
| 1007 | 6 | 42 | 66 | | 14 | 77 | | 67 | 75 | |
| 1008 | 40 | 71 | 71 | | 77 | 78 | | 81 | | |
| 1009 | 0 | 45 | 71 | | 39 | 80 | | 79 | | |
| 1010 | 1 | 16 | 64 | | 17 | 76 | | 77 | | |
| 1011 | 2 | 35 | 68 | | 51 | 85 | | 65 | 78 | |
| 1012 | 3 | 59 | 75 | | 37 | 82 | | 71 | 80 | |
| 1013 | 7 | 60 | 58 | | 37 | 65 | 64 | 80 | | |
| 1014 | 3 | 60 | 61 | | 65 | 72 | 75 | 58 | 77 | |
| 1015 | 8 | 17 | 18 | 19§ | 3 | 64 | 62 | 84 | | |

*The 7 vitamins as listed in Table II.

†The purines and pyrimidines used were adenine, guanine, xanthine, hypoxanthine, thymine, and uracil. Each compound was added in amount of 10 μg. per ml.

‡Heavier growth later for this culture: turbidity reading of 61 at 7 days' incubation.

§Heavier growth of Culture 1015 did not appear on 7 days' incubation.

The yeast extract was sterilized by filtration.

*Effect of Purines, Pyrimidines, and Other Substances.*—The results presented up to this point were obtained without the addition of purines and pyrimidines. If these substances are added there is a marked stimulation in the rate of growth of many cultures. This may be seen from Table IV which shows the effect of these compounds when added with the vitamins. Growth of many cultures was appreciably affected and approximately one-half of them

attained moderate or good growth within the first 24 hours. Certain individual cultures were markedly stimulated, for example cultures 4, 26, 641, 1002, 1003, 1006, and 1015; others were stimulated in less degree and a few showed little response to the added compounds. In the latter category are Cultures 9, 19, 1004, and 1008.

The effect of the purines, pyrimidines, and glutamine appears for the most part to be purely a stimulatory one. These substances are not a definite growth requirement for any of the cultures used in this study with the possible exception of Culture 1015. Though they often accelerate growth, the final luxuriance of growth is frequently not much greater than that eventually reached by the cultures which received only the vitamin supplement. Also, these compounds when added to the basal medium without vitamins do not support growth, i.e., they are not capable of replacing needed vitamins. This was shown in separate experiments which are not given in the table.

TABLE V

EFFECT ON GROWTH OF THE INDIVIDUAL COMPOUNDS AND OF COMBINATIONS OF PURINES, PYRIMIDINES, AND GLUTAMINE, SUPPLIED WITH NEEDED VITAMINS

| COMPOUNDS | TURBIDITY READINGS OF LACTOBACILLUS CULTURES | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 3 HOURS | | | 4 HOURS | | | 96 HOURS | |
| | 24 | 48 | 72 | 24 | 48 | 72 | 24 | 48 |
| 1. Basal medium | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| 2. Basal medium plus 6 vitamins | 3 | 54 | 73 | 0 | 9 | 75 | 8 | 82 |
| 3. No. 2 plus adenine* | 5 | 63 | 74 | 5 | 71 | 80 | 16 | 82 |
| 4. No. 2 plus thymine | 6 | 63 | 74 | 2 | 30 | 69 | 6 | 81 |
| 5. No. 2 plus uracil | 2 | 67 | 74 | 5 | 65 | 70 | 4 | 80 |
| 6. No. 2 plus thymine and uracil | 2 | 66 | 73 | 2 | 69 | 75 | 4 | 80 |
| 7. No. 2 plus adenine and thymine | 5 | 68 | 74 | 8 | 66 | 80 | 17 | 82 |
| 8. No. 2 plus adenine, thymine, and glutamine | 9 | 67 | 74 | 9 | 78 | 80 | 33 | 81 |
| 9. No. 2 plus guanine and thymine | 5 | 64 | 73 | 11 | 79 | 80 | 11 | 82 |
| 10. No. 2 plus hypoxanthine and thymine | 4 | 68 | 77 | 8 | 81 | 83 | 15 | 81 |
| 11. No. 2 plus adenine, guanine, xanthine, and hypoxanthine | 4 | 65 | 76 | 38 | 80 | 83 | 20 | 82 |
| 12. No. 11 plus thymine, uracil, and glutamine | 11 | 74 | 76 | 60 | 82 | 85 | 44 | 80 |

*Each compound was added in the amount of 10 μg. per ml. of medium.

A comparison of the growth with that supported by addition of 0.2 per cent yeast extract to the basal medium is shown also in Table IV. In the presence of the yeast extract almost all of the oral lactobacilli attained maximum growth in approximately the first 24 hours. Growth response was more prompt than with the vitamins and was also somewhat speedier than in the presence of the vitamins, purines, pyrimidines, and glutamine. Evidently yeast extract contains other substances which accelerate growth of these organisms. There is some reason for believing, too, that some of these other substances are heat labile, for when the yeast extract was sterilized by autoclaving, rather than filtration, growth of 2 of the lactobacilli was definitly poorer.

Table V shows the effect of single compounds and of various combinations on the rate of growth of 3 of the cultures. These 3 cultures are about as representative a sample as it is possible to select from a total of 26 when the re-

sults differ somewhat in detail from one culture to another and from compound to compound. Culture 3 grew moderately well in the presence of the vitamins and was stimulated but little by the added purines and other compounds (compare line 2 with lines 3 to 12 of Table V). Culture 4 was stimulated appreciably by additions of single compounds or combinations of 2 or 3 of them, and markedly stimulated by the mixture of all of them (compare the 24- and 48-hour growth shown in line 2 with that of lines 3 to 10 and lines 11 and 12). Growth of Culture 96 was stimulated moderately by combinations of several compounds (compare especially the results in lines 2, 3, 7, 8, 11, and 12).

The effect of the compounds was in general largely an additive one, a number of them usually affording more of a stimulus than any one or combination of 2 or 3. Also the effect of these compounds often was not specific, one substance substituting for another in producing stimulation of growth. With many strains of oral lactobacilli, either adenine, guanine, or hypoxanthine produced much the same growth stimulus. Occasionally, in contrast to such findings, one or another of these compounds seemed to be slightly inhibitive. Such an effect, however, was noted only infrequently and was never marked. It seemed to be masked by the addition of other related compounds when a mixture of several compounds was used.

Concerning the results with thymine it will be recalled that the basal medium contained pteroylglutamic acid (folic acid) and that any effect of thymine, which may substitute for folic acid in the growth of some microorganisms, would not be so likely to appear under these conditions. We obtained no evidence of a definite need for thymine in addition to pteroylglutamic acid.

The interchangeability of a number of purines and pyrimidines, as noted in this study, has been observed before. It may vary with different organisms, being more evident with some than with others. Snell and Mitchell[17] have reported some results of this nature for *Lactobacillus arabinosus, L. pentosus, Leuconostoc mesenteroides,* and *Streptococcus faecalis.* Merrifield and Dunn[18] examined a total of 123 bacteria for pyrimidine requirement and found that only 6 of them showed definite or specific requirements for these compounds; among the other organisms the effect of one compound could usually be replaced by another.

Glutamine, the amide of glutamic acid, in only a few cases seemed to exert some stimulative effect. It will be recalled that one of the chief ingredients of the basal medium was hydrolyzed casein and that the medium, therefore, must have contained considerable glutamic acid. Many organisms such as *L. casei, L. arabinosus,* and *Str. faecalis* may respond to glutamic acid as well as to glutamine.[19] In the presence of adequate amounts of glutamic acid an effect of glutamine might not become apparent; or stated differently, either glutamic acid or glutamine would satisfy the requirement.

*Tween 80.*—Oleic acid, some related fatty acids, and certain lipides often are capable of replacing biotin for growth of lactobacilli and other microorganisms which ordinarily require biotin.[20-23] Furthermore, the oleate or related substance at times may be needed in addition to biotin. Williams, Broquist, and Snell[22] noted that several lactobacilli, tentatively identified as *L, acidophilus, L.*

*bulgaricus,* and *L. helveticus* required oleates or related compounds even in the presence of biotin, and Whitehill, Oleson, and Subbarow[24] obtained a lactobacillus from rat intestine which also possessed a definite requirement for oleate.

Since a few of our oral lactobacilli grew slowly or poorly in the tests with known vitamins, including biotin, an oleate water-soluble compound was tried in additional tests, particularly with some of the slower-growing cultures, to see if it would stimulate growth. Tween 80, a sorbitan monoleate polyoxyalkylene derivative, was employed in amounts of 100 μg. and 1000 μg. per ml. of medium. Before use it was purified by the method of Davis[25] to reduce the content of free fatty acid which may be sufficiently high to cause some bacteriostatic effect. The purified product was added to the basal medium plus vitamins, including biotin, and also to the basal medium plus vitamins, purines, and pyrimidines.

Tween 80 produced little effect on growth of the majority of cultures used in these tests, either in rapidity of cell multiplication or final luxuriance. With a few cultures some moderate stimulating effect was seen, but this was not especially prominent and in a few other cases there seemed to be a slight retarding effect on the rate of growth. In no instance was the oleate compound needed for growth in the presence of biotin.

*Comparison of Recently Isolated and Older Cultures.*—In general, there were no marked differences in the vitamin requirement of recently isolated strains and those which had been maintained in the laboratory for a number of years before the tests were made. The only exception was Culture 1015 (recently isolated) which grew poorly in the presence of vitamins, but responded to the combination of purines, pyrimidines, and vitamins (Table IV). Evidently this culture has a more specific requirement for one or more of the purines or pyrimidines, but the detailed requirement was not investigated further at this time.

## SUMMARY

The vitamin requirement of 26 strains of oral lactobacilli was studied. These cultures were obtained from carious lesions, plaques, and saliva; 11 of them were laboratory stock cultures, the other 15 were recently isolated.

From 4 to 6 vitamins were required by the different cultures for growth in a basal medium of casein digest, glucose, acetate, and salts. Although this growth was slower than in media containing products such as yeast extract, it could be carried through successive transfers in the presence of the needed vitamins.

All of the oral lactobacilli required nicotinic acid, pantothenic acid, and biotin. Many of them required vitamin $B_6$ (supplied as pyridoxamine), or pteroylglutamic acid, or both. The need for vitamin $B_6$ and pteroylglutamic acid, when it occurred, was not always an absolute one, some strains developing poorly without one or the other of these compounds. Seventeen of the cultures required riboflavin and not thiamine, while 8 others required thiamine and not riboflavin.

A thiamine requirement was associated with the heterofermentative type of glucose metabolism, a finding in agreement with the previous report of others.

There were no essential differences in vitamin requirement between the older laboratory cultures as a group and the more recently isolated ones.

Purines and pyrimidines when added with the vitamins often accelerated the rate of growth, in some instances quite markedly. A mixture of several purines and pyrimidines produced a greater stimulation of growth than the individual compounds.

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# SALIVARY MUCIN AS A SOURCE OF ESSENTIAL AMINO ACIDS FOR THE LACTOBACILLUS ACIDOPHILUS (HADLEY)

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IN 1946, Weisberger and Johnson[1] demonstrated that casein hydrolysate and added tryptophane were required for the maximum growth and acid production of an oral strain of *Lactobacillus acidophilus* when this organism was cultured in the chemically defined synthetic medium described by Landy and Dicken.[2] Subsequently, Weisberger[3, 4] found that whole saliva hydrolyzed by acid or alkali could replace casein hydrolysate, and whole saliva incubated with glucose at 37.5° C. for 24 hours or longer could be substituted for added tryptophane in the synthetic medium. Of the amino acid constituents of casein hydrolysate, human saliva contains all but alanine and aspartic acid.[5, 6] It would seem, therefore, that saliva may serve as a source of essential amino acids for the oral *L. acidophilus*. This postulate is supported by the recent work of Rogers[7] who found that salivary mucin, a glycoprotein rich in amino acids and carbohydrates, may undergo bacterial hydrolysis and that the products of the hydrolysis may constitute a source of nutrients for some bacteria. Proteolytic enzymes capable of hydrolyzing salivary mucin may be elaborated in the oral cavity by disintegrating leucocytes[8] and by proteolytic microorganisms.[9] Since mucin may be a constituent of the dental plaque, it was decided to determine (1) whether an oral strain of lactobacillus associated with dental caries could utilize salivary mucin as a replacement for tryptophane and/or casein hydrolysate in a synthetic medium containing all of the other known growth requirements of this organism, and (2) whether an exogenous source of proteolytic, mucolytic and/or amylolytic enzyme is required for this process.

## MATERIALS AND METHODS

Salivary mucin was prepared by adding filtered, pooled, paraffin-stimulated saliva to 4 volumes of 95 per cent ethyl alcohol with constant stirring. The resulting suspension was permitted to stand in a covered vessel for at least 12 hours. It was then filtered and the precipitated mucin washed with 95 per cent ethyl alcohol and ether. The mucin was carefully recovered, dried, and ground into a fine powder.

The test medium consisted of that described by Landy and Dicken[2] (Table I). It was modified for the purpose of this study by omitting added tryptophane from Assay I, casein hydrolysate from Assay II, and both added tryptophane and casein hydrolysate from Assay III. These constituents were replaced by precipitated salivary mucin in equal amounts by weight. The contents of each tube in each assay are listed in Table II. Salivary mucin was added in the amount of 2 mg. per tube in Assay I, 50 mg. per tube in Assay II, and 52

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mg. per tube in Assay III before autoclaving. Trypsin, amylopsin, and hyaluronidase were added in amounts of 1 mg. each per tube after autoclaving (Table II). Each milligram of hyaluronidase, equivalent to 150 turbidity reducing units, was supplied with 2 mg. of polypeptide derived from gelatin which acted as a stabilizer for the enzyme.

TABLE I

COMPOSITION OF UNMODIFIED TEST MEDIUM*

| CONSTITUENT | QUANTITY |
|---|---|
| Casein hydrolysate | 2.5 ml. |
| Salt solution A† | 0.5 ml. |
| Salt solution B‡ | 0.5 ml. |
| Glucose | 1.0 Gm. |
| Sodium acetate | 600.0 mg. |
| Asparagine | 25.0 mg. |
| Tryptophane | 10.0 mg. |
| Cystine | 10.0 mg. |
| Guanine hydrochloride | 500.0 μg. |
| Adenine sulfate | 500.0 μg. |
| Xanthine | 500.0 μg. |
| Uracil | 500.0 μg. |
| Pyridoxine hydrochloride | 40.0 μg. |
| Riboflavin | 20.0 μg. |
| Calcium pantothenate | 20.0 μg. |
| Nicotinic acid | 20.0 μg. |
| Thiamine hydrochloride | 10.0 μg. |
| Folic acid | 1.0 μg. |
| Biotin | 0.5 μg. |
| Distilled water to 50 ml. | |

*Double strength, Landy and Dicken medium adjusted to pH 6.8 with NaOH.

†Salt Solution A:
$K_2HPO_4$ 5 Gm.
$KH_2PO_4$ 5 Gm.
Distilled water to 50 ml.

‡Salt Solution B:
$MgSo_4.7\ H_2O$ 10.0 Gm.
NaCl 0.5 Gm.
$FeSO_4.7\ H_2O$ 0.5 Gm.
$MnSO_4.2\ H_2O$ 0.337 Gm.
Distilled Water to 250 ml.

The Hadley strain of oral *L. acidophilus* (ATCC #4646) obtained from a carious lesion served as the test organism. One drop of inoculum, the preparation of which has been described in detail in a previous publication,[10] was added to each tube in each assay after autoclaving, cooling, and adding the enzymes as shown in Table II. Each assay was set up in duplicate. Inoculated and uninoculated tubes of unmodified Landy and Dicken medium constituted the controls. The tubes were incubated at 37° C. for 72 hours. Growth was measured by centrifuging the cultures in Hopkins vaccine tubes for 10 minutes at high speed and then immediately reading the packed cell volume. Acid production was determined by titrating the centrifugates with 0.1N NaOH to the pH of the uninoculated control tubes. A Beckman pH meter was used for this purpose.

## OBSERVATIONS

*Assay I* (*Added tryptophane withheld from the test medium*).—Fig. 1 shows the effect of the replacement of added tryptophane in the test medium by an equal amount by weight of salivary mucin (2 mg.), and/or 1 mg. each of the proteolytic, mucolytic, and amylolytic enzymes on the growth and acid production of the test organism. Minimal growth was observed in the tube containing no substitute for the omitted tryptophane and in those containing

salivary mucin, hyaluronidase, and both salivary mucin and hyaluronidase. The acid content of the tubes containing trypsin and amylopsin was equivalent to 30.0 per cent and 27.7 per cent, respectively, of that present in the control tube. In the tubes containing salivary mucin in addition to trypsin and amylopsin, acid production equaled 60.8 per cent and 36.3 per cent of the control, respectively. The greatest amount of acid, equivalent to 81.7 per cent of the control, was formed in the tube containing salivary mucin, trypsin, amylopsin, and hyaluronidase.

TABLE II

SALIVARY MUCIN AND/OR TRYPSIN, AMYLOPSIN, AND HYALURONIDASE AS SOURCES OF ESSENTIAL AMINO ACIDS FOR THE GROWTH OF THE LACTOBACILLUS ACIDOPHILUS (HADLEY)*

| TUBE CONTENTS (TEST MEDIUM) | ASSAY I (TRYPTOPHANE OMITTED) | | ASSAY II (CASEIN HYDROLYSATE OMITTED) | | ASSAY III (TRYPTOPHANE AND CASEIN HYDROLYSATE OMITTED) | |
|---|---|---|---|---|---|---|
| | GROWTH (PACKED CELL VOL.)† | ACID PRODUCTION (C.C. 0.1N NaOH) | GROWTH (PACKED CELL VOL.)† | ACID PRODUCTION (C.C. 0.1N NaOH) | GROWTH (PACKED CELL VOL.)† | ACID PRODUCTION (C.C. 0.1N NaOH) |
| 1. Unmodified | +++ | 13.65 | +++ | 13.65 | +++ | 13.65 |
| 2. + Mucin, amylopsin, trypsin, and hyaluronidase | +++ | 11.15 | +++ | 10.68 | +++ | 10.36 |
| 3. + Mucin and trypsin | +++ | 8.30 | +++ | 8.45 | +++ | 8.41 |
| 4. + Mucin and amylopsin | +++ | 4.95 | +++ | 7.64 | +++ | 7.36 |
| 5. + Trypsin | +++ | 4.10 | + | 1.60 | + | 1.65 |
| 6. + Amylopsin | ++ | 3.10 | + | 0.85 | + | 0.90 |
| 7. + Mucin and hyaluronidase | + | 0.75 | ++ | 3.13 | ++ | 3.30 |
| 8. + Mucin | + | 0.65 | ++ | 3.08 | ++ | 3.17 |
| 9. + Hyaluronidase | + | 0.65 | - | 0.00 | - | 0.00 |
| 10. - Tryptophane and/or casein hydrolysate | + | 0.50 | - | 0.00 | - | 0.00 |

*72-hour reading.
†0.0 - 0.1 c.c. -
0.1 - 0.25 c.c. ±
0.25- 0.5 c.c. +
0.5 - 1.0 c.c. ++
1.0 - 2.0 c.c. +++

*Assay II* (*Casein hydrolysate withheld from the test medium*).—The effect of withholding casein hydrolysate from the test medium and its replacement by salivary mucin in an equal amount by weight (50 mg. per tube) with and without the various enzymes is also shown in Fig. 1. No growth or acid production was noted in the tube containing no replacement for casein hydrolysate and in the tube to which hyaluronidase was added. Acid in amounts ranging from 6.2 per cent to 22.9 per cent of that formed in the control tube was produced in the tubes containing trypsin, amylopsin, mucin, and both mucin and hyaluronidase. Submaximal acid production was observed in the tubes containing mucin and trypsin, mucin and amylopsin, and mucin and the 3 enzymes. The amounts of acid formed were equivalent to 61.9 per cent, 56.0 per cent and 77.5 per cent, respectively, of that present in the control tube containing the complete Landy and Dicken medium.

*Assay III* (*Tryptophane and casein hydrolysate withheld from the test medium*).—The results of the omission of both tryptophane and casein hydrol-

ysate from the test medium and their replacement by an equal amount by weight of mucin (52 mg.) and by the various enzymes were similar in magnitude to those noted in Assay II. No growth occurred in the tube containing no replacement and in the tube containing hyaluronidase. Small amounts of acid were formed in the tubes containing trypsin, amylopsin, mucin, and both mucin and hyaluronidase. Submaximal acid production equivalent to 61.6 per cent, 53.9 per cent, and 75.0 per cent of the control occurred in the tubes containing mucin and trypsin, mucin and amylopsin, and mucin and the 3 enzymes, respectively.

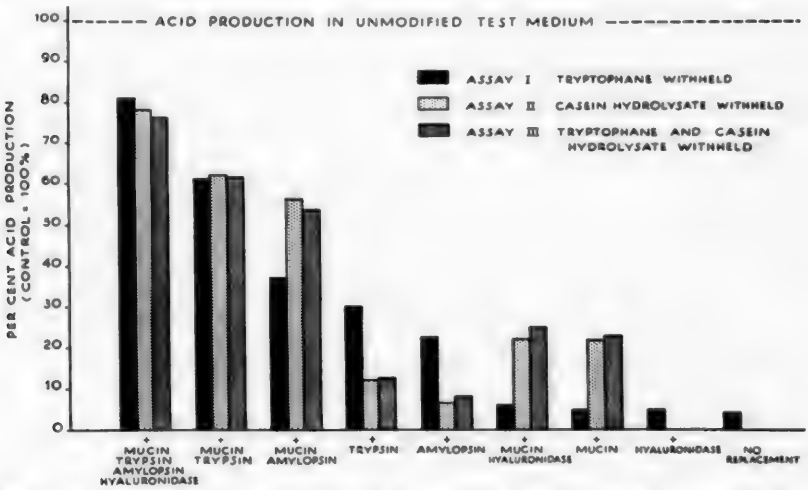


Fig. 1.—The effect of the replacement of tryptophane and/or casein hydrolysate by salivary mucin and/or certain enzymes on acid production by *L. acidophilus* (Hadley).

## DISCUSSION

It has been suggested that the mucin and mucinate contained in the dental plaque may serve as a source of nourishment for the organisms retained therein.[11] Lothrop[12] has shown that salivary mucin is precipitated at acid concentrations capable of dissolving enamel. In this study it was found that in the absence of amylolytic or proteolytic enzymes, precipitated salivary mucin is a poor source of the amino acids required for the maximal growth of an oral strain of the *L. acidophilus* in a synthetic medium containing all of the other known growth requirements of this organism. Since mucin contains fairly large amounts of tryptophane and other amino acids,[13] it would seem that the amino acids contained in mucin are present either in an unavailable form, in insufficient quantities, or both. This hypothesis was substantiated by the demonstration that the effectiveness of mucin as a replacement for added tryptophane and/or casein hydrolysate was substantially increased by the presence of trypsin and/or amylopsin in the test medium. Since both of these enzymes were also utilized by the test organism to a slight extent in the absence of mucin when tryptophane and/or casein hydrolysate were withheld from the medium, it would appear that these enzymes served both as a source of nutrients and as an

adjunct whereby some of the amino acid constituents of mucin were made available for use by the test organism. These findings aid in interpreting the results of Fosdick and Rapp[14, 15] who found that the addition of trypsin or amylopsin stimulated acid production in saliva-glucose-powdered enamel mixtures.

The possibility that enzymes or constituents of enzymes secreted by tissues or by bacteria may serve as growth factors for other bacteria in the oral cavity is one which is of extreme interest and one which may serve to explain some of the hitherto unsolved problems relating to the mechanism of dental caries.

## SUMMARY AND CONCLUSIONS

1. Precipitated salivary mucin, although rich in amino acids, is a poor replacement for tryptophane and/or casein hydrolysate in the Landy and Dicken medium which, when unmodified, is nutritionally adequate for the maximum growth of the Hadley strain of oral *L. acidophilus*.

2. The value of precipitated salivary mucin as a source of essential amino acids for the test organism was substantially enhanced by the addition of crystalline trypsin and/or amylopsin to the test medium from which casein hydrolysate and/or tryptophane was omitted.

3. Precipitated salivary mucin may serve as an efficient source of essential amino acids for the oral *L. acidophilus* in the presence of an exogenous source of proteolytic or amylolytic enzymes capable of hydrolyzing this glycoprotein.

We wish to thank Dr. David Klein of the Wilson Laboratories, Chicago, Ill., for the trypsin and amylopsin and the Schering Corporation, Bloomfield, N. J., for the hyaluronidase used in this study.

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# AMINO ACIDS IN SALIVA OF HUMAN BEINGS ON A LOW PROTEIN DIET

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THE presence of free amino acids in human saliva has been reported.[1] At that time no attempts were made to control the diet of the individuals who volunteered to supply the saliva. It was found also that the variations were independent of caries activity.

During the past two years we had an opportunity to collaborate in a nutritional study which is conducted under the direction of Dr. Janice Smith in the Department of Home Economics on the Urbana Campus of the University of Illinois. Seven university women volunteered for this study for the school year and were provided with living quarters and board as well as a stipend for participating in this program. Of the 7 original subjects that started the project, the first year, 4 remained for the full 8-month period while out of a group of 6 (different subjects) starting the second year, 5 remained for the full test period.

The basal nitrogen diet varied from a low of 0.2 Gm. of nitrogen per person to 3.4 Gm. depending upon a particular test period. The diet was changed every 10 days as far as the nitrogen content was concerned. Forty-five calories were provided per kilogram of body weight. The nitrogen-free part of the diet consisted of sugar, corn starch, salt mixture, vitamins, lemon juice, and agar, while the protein mixture consisted of oatmeal, ground beef, 18 per cent cream, potatoes, whole eggs, and white bread made with water.

Our interest in the study centered around the effect, if any, a low protein diet may have on the amino acid composition of the saliva. Specimens of saliva were collected before breakfast by paraffin stimulation. Since the diet was changed every 10 days, the saliva specimens were taken 1 or 2 days prior to this change in the diet, so that the subjects had been on a particular diet for at least 8 days.

The saliva was autoclaved immediately after collection at a pH of about 6.5 so that hydrolysis of the protein would not take place. The samples were assayed for the free amino acids within 24 hours after collection. Certain limitations would not allow the analysis of more than 4 amino acids during each year. Leucine, methionine, histidine, and tryptophane analyses were made on the saliva provided by the first group, while proline, cystine, arginine, and serine determinations were carried out on the saliva of the second group. The methods of assay used were the same as those previously reported.[1] Only the free amino acids have been determined. No attempts were made to analyze for the combined forms.

Figs. 1 to 8 show the salivary content of the 8 amino acids during the various test diet periods.

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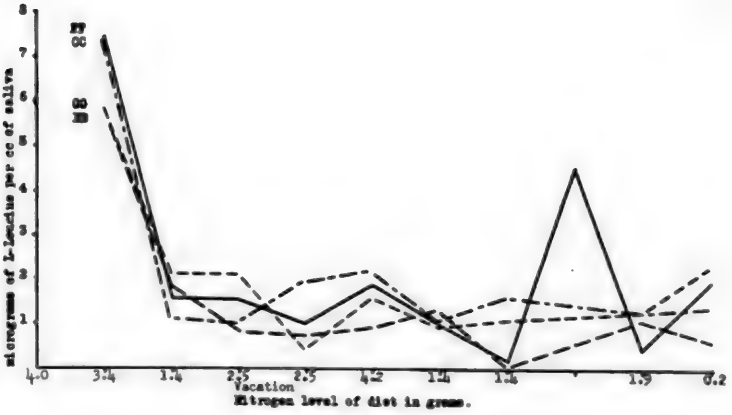


Fig. 1.

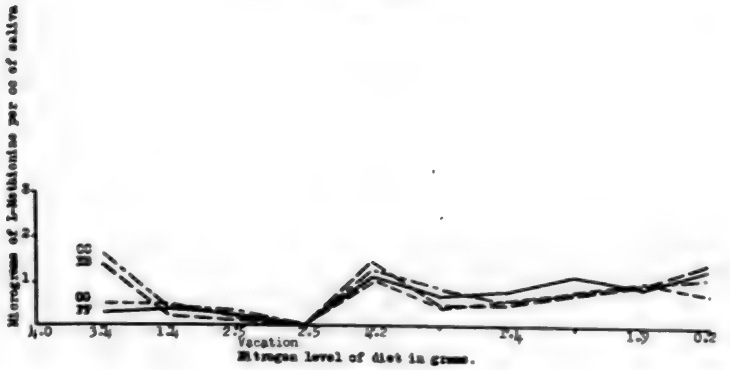


Fig. 2.

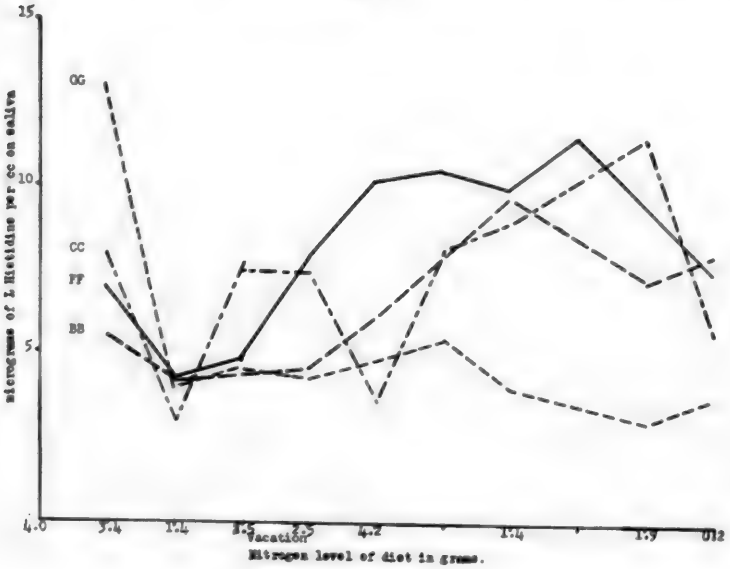


Fig. 3.

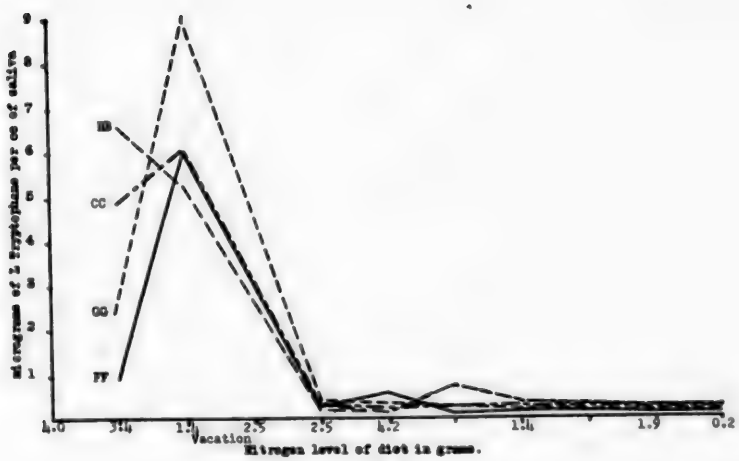


Fig 4.

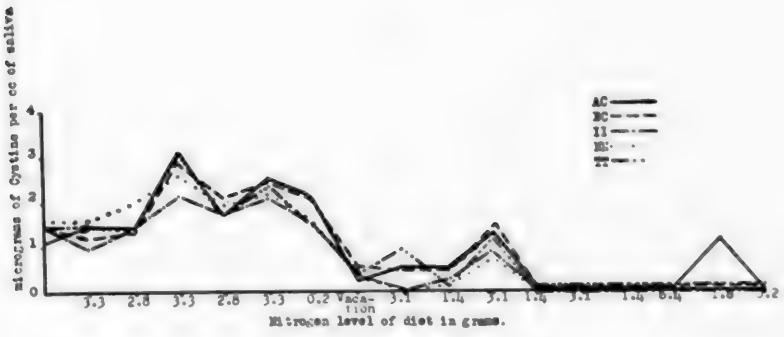


Fig. 5.

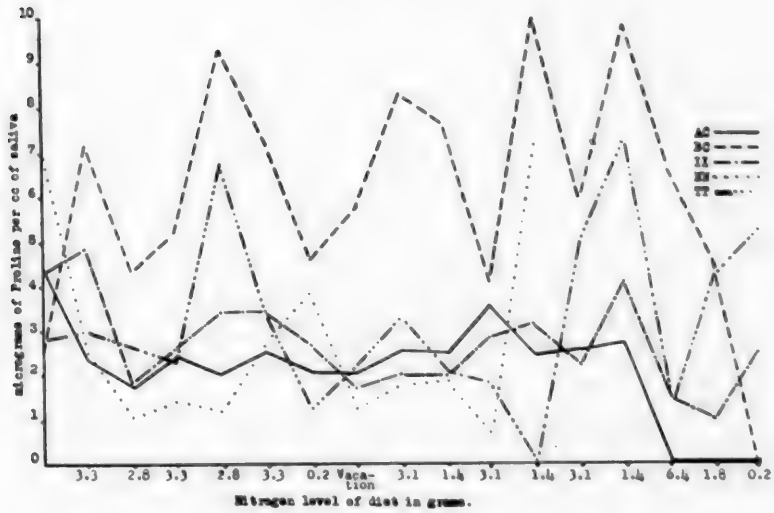


Fig. 6.

## DISCUSSION

The values of the amino acids reported here are for the free acids only. No attempts were made at this time to determine the conjugated forms. No correlation between caries activity and the amino acid content of the saliva could be established. As can be seen from Figs. 1 to 4, one of the individuals, G. G.,

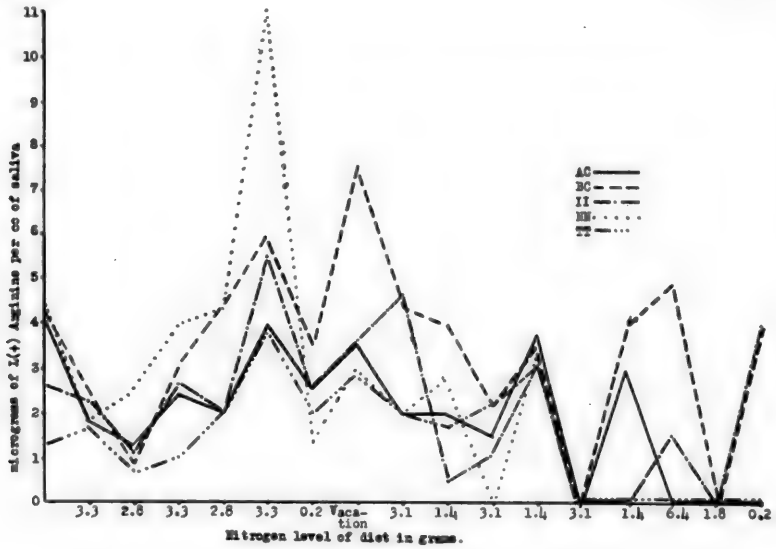
Micrograms of l(+) Arginine per cc of saliva
AC
BC
II
NN
TT
3.3 2.8 3.3 2.8 3.3 0.2 Vacation 3.1 1.4 3.1 1.4 3.1 1.4 6.4 1.8 0.2
Nitrogen level of diet in grams.

Fig. 7.

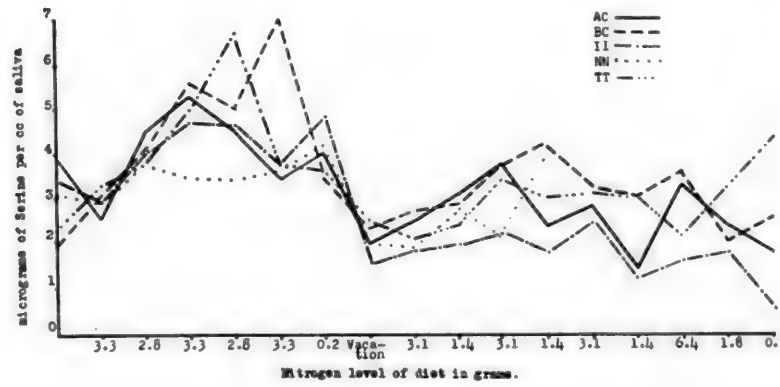
Micrograms of Serine per cc of saliva
AC
BC
II
NN
TT
3.3 2.8 3.3 2.8 3.3 0.2 Vacation 3.1 1.4 3.1 1.4 3.1 1.4 6.4 1.8 0.2
Nitrogen level of diet in grams.

Fig. 8.

who was caries inactive at the time of the tests, followed the same pattern of variations as far as the fluctuations of leucine, methionine, histidine, and tryptophane. Similar results were obtained on the saliva of the second group. These latter samples were assayed for cystine, proline, arginine, and serine and the results are shown in Figs. 5 to 8.

Bergeim[2] studied the urinary excretion of amino acids of these same individuals during the first test period. He found that with the exception of phenylalanine and tyrosine there was a decrease in the amount of these acids excreted by the subjects on a low protein diet as compared to those on a normal diet. A direct correlation between intake and amino acid excretion does not exist, since the urinary excretion on a 0.2 Gm. of nitrogen per day is not different from that on a 2.5 Gm. of nitrogen per day.

In our studies, all of the amino acid levels were highest at the start of the diet period. In the case of histidine and methionine the levels fell to about one-half of their original values, while a more pronounced drop was observed in the case of leucine. The values for tryptophane changed the most. While they were relatively low at the start of the diet period, they increased manyfold during a vacation period when an unrestricted diet was allowed. They fell to very low levels again during the rest of the period of low nitrogen intake.

While no correlation can be drawn at the present time between the amino acid content of the saliva and the low nitrogen dietary intake, it is significant that the amino acids are present in human saliva in measurable quantities even when a person is on a low nitrogen diet.

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# SOME ANTAGONISTIC ACTIVITY OF BACTERIA FROM THE HUMAN ORAL CAVITY

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## REVIEW OF LITERATURE

AT THE turn of the century, bacteriologic studies had shown that a host of diseases were caused by specific organisms gaining entry into the body. It had been shown also that many of these diseases also were prevented by an immunity mechanism. It was natural for investigators to turn their attention toward the mechanism whereby the oral cavity normally rendered innocuous the host of pathogenic forms with which it came in contact. Hugenschmidt's work[1] is illustrative of the early work in this connection. Among the several possible ways a wound in the oral cavity was prevented from becoming infected, he recognized the possible antagonism of microorganisms in the oral cavity.

Miller[2] continued the investigation of possible antiseptic properties of saliva against pathogenic forms and found, on the basis of adding *Bacillus prodigiosus* to saliva in the mouth, that there was slight chance of an outside organism being able to survive against the normal oral flora. The bactericidal properties of the saliva itself were not detected by his methods.

Thirty years later, a group of investigators at the University of Rochester made a more complete analysis of the lethal action of saliva on bacterial forms by using the well technic. Clough,[3] in addition to demonstrating the lethal action on pathogenic forms, showed that 40 of 41 different salivas produced a zone of lactobacillus inhibition around the well containing saliva. He also stated that filtered saliva showed no inhibition. However, in subsequent articles the group,[3-7] by using Berkefeld filtration, centrifugation, and broth cultures and testing the various fractions, came to the conclusion, with the possible exception of *Micrococcus lysodeikticus,* that the active principle was in the saliva and not in the oral bacteria. The inhibition was greatest for water and air bacteria and the least affected were those normally found in the oral cavity.

This saliva-inhibiting property was considered by Hill[8, 9] to be greater against lactobacillus in caries-resistant salivas than in caries-susceptible salivas. Later, Hill, White, Matt, and Pearlman,[10] reported the extraction of an active principle from the saliva.

Another somewhat different line of investigation has involved acidophilic organisms being used to combat various oral infections. According to the literature review by Appleton,[11] in general only slight effect has been produced.

This investigation was supported by a research grant from the National Institute for Dental Research of the National Institutes of Health, United States Public Health Service, Bethesda, Md.

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The principal investigations to date indicate that the inhibitory property of saliva is inherent in the saliva itself and is not a property of the oral bacteria. The bacterial inhibitory action on a few pathogenic forms have been reported and reviewed adequately by Van Kesteren, Bibby, and Berry,[7] and Hill.[8] The most recent work by White and Hill,[12] using the well technic, found several organisms that could be inhibited by lactobacillus, but *Pseudomonas aeruginosa* was the only organism found which would inhibit *Lactobacillus acidophilus*. The work to be described, where the agar streak-plate test was used, would seem to indicate the bacterial antagonism is more prevalent than has been previously described.

## MATERIALS

Two saliva pools were used as the source material for the oral bacteria. One pool was collected from caries-free persons and the other from individuals having ten or more open carious lesions. The two saliva pools were kept refrigerated from the time of collection until the plates were inoculated.

Three strains of lactobacilli were used: one secured from saliva, the Hadley No. 4646 secured from the American type culture collection, and a lyophilized strain secured from the Agriculture Regional Laboratory at Peoria, Ill.

The culture media for securing the random sample of oral bacteria from the saliva consisted of nutrient agar, blood agar, blood agar base, dextrose agar, corn meal agar, sugar agar (0.5 per cent sucrose, 0.5 per cent lactose, 0.5 per cent glucose), Sabouraud's agar, yeast dextrose agar, tomato juice agar, tooth extract agar, trypsin digest agar, nutrient broth pH 5, and nutrient broth pH 6.8.

For the streak-plate tests, yeast dextrose agar was used, because most of the isolated organisms and the lactobacilli could be grown easily on this medium.

The medium for the tooth fragment weights and calcium determinations was pH 6.8 nutrient broth with 5 per cent sucrose. The tooth fragments consisted of dentin, the approximate size of wheat grains.

## METHODS

To obtain the random sample of oral bacteria antagonistic to the lactobacilli, Petri dish culture plates were prepared with the 13 types of culture media and inoculated by streaking over the entire surface with the following saline dilutions of the immune and susceptible saliva: 1:1, 1:10, 1:100, 1:1000. Thus, each media type had 4 plates (52 plates in all) and a grand total of 104 plates were inoculated.

The inoculated culture plates were incubated aerobically at 37° C. and examined at 24, 48, and 72 hours. As the bacterial colonies started appearing, each that appeared different in any discernible way was transferred to the same type of media from which it was secured and subsequently isolated in pure culture. In this way 30 pure cultures were established, 17 from the susceptible and 13 from the immune salivas.

Each of the 30 pure cultures was tested for antagonistic action against 3 lactobacilli by using the agar streak-plate method.[13] The pure culture of the

test organism was streaked across the yeast dextrose agar plate and incubated for 72 hours at 37° C. Then each of the 3 lactobacilli was streaked across the plate into the test organism streak at right angles. The plate was then re-incubated for 96 hours at 37° C. and the distance, if any, from the test organism growth where no lactobacillus growth occurred measured (Fig. 1). Since the antagonistic action was the object of the study, only those showing some lactobacillus inhibition were retained and classified. Although almost all the bacteria grew readily on the yeast dextrose agar media, in a few instances no media could be found where the culture would grow; hence, a few additional pure cultures were lost. This left a total of 14 that were tested further. These 14 pure cultures were tested with the agar streak-plate procedure as described above for their ability to inhibit each other.*

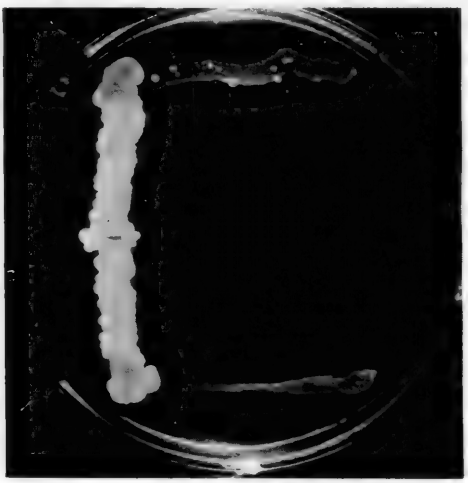

Fig. 1.—Agar streak-plate method for testing bacterial antagonism. The vertical line of growth is the test organism. The organism in the bottom horizontal line of growth was inhibited slightly; the middle was considerably inhibited and the top organism was not inhibited at all.

The next test consisted of determining the ability of each test organism to prevent dentin fragment weight loss when subjected to lactobacillus in nutrient broth. Teeth, immediately after extraction, were washed, scraped, placed in wet gauze, and frozen until used. At that time they were removed from the refrigerator for thawing. The teeth were then broken into small pieces, after being wrapped in Cellophane, by being pounded with a pestle in a mortar. Approximately 10 pieces of dentin weighing 200 to 300 mg. were used per sample bottle. Enough distilled water was added to each bottle to barely cover the dentin fragments and the bottles containing the fragments were autoclaved. The remaining procedures were carried out with aseptic technic.

The bottles with the dentin fragments covered with water were again refrigerated, not over 48 hours. The 10 pieces of dentin from each sample bottle

*The authors are indebted to Mrs. Adrienne Dattner for much of the bacteriologic culture work.

were placed between sterile filter paper, oven-dried at 100° C. for 10 minutes, and weighed on a chainomatic analytical balance. The weighed dentin fragments were then placed in a bottle containing 15 c.c. of nutrient broth with 5 per cent sucrose, 0.1 c.c. saline suspension of Hadley lactobacillus, and 0.1 c.c. saline suspension of the test organism. Twelve bottles prepared in this way were incubated at 37° C. for 4 hours, another 12 for 24 hours, an additional 12 for 48 hours, and still another 12 for 72 hours. At the termination of the respective incubation periods, the dentin fragments were removed, oven-dried at 100° C., and again weighed. The difference between the final and original weight was determined and the percentage of gain or loss recorded.

TABLE I

LACTOBACILLUS INHIBITION BY VARIOUS ORGANISMS FROM THE SALIVA

| NUMBER | NAME | LACTOBACILLI | | |
|---|---|---|---|---|
| | | SALIVA | LYOPHILIZED | HADLEY |
| $S_1$ | | None | None | None |
| $S_2$ | *Micrococcus varians* | Complete | Complete | Complete |
| $S_3$ | | None | None | None |
| $S_4$ | | None | None | None |
| $S_5$ | Died | | | |
| $S_6$ | | None | None | None |
| $S_7$ | *Micrococcus varians* | Complete | Complete | 1 inch |
| $S_8$ | | None | None | None |
| $S_9$ | *Escherichia freundii* | Complete | Complete | Complete |
| $S_{10}$ | Mold | Complete | Complete | Complete |
| $S_{11}$ | | None | None | None |
| $S_{12}$ | *Bacillus subtilis* group | Complete | Complete | Complete |
| $S_{13}$ | *Bacillus brevis* group | Complete | Complete | Complete |
| $S_{14}$ | *Bacillus brevis* group | Complete | Complete | Complete |
| $S_{15}$ | *Bacillus subtilis* group | Complete | Complete | Complete |
| $S_{16}$ | | $\frac{1}{16}$ inch | None | None |
| $S_{17}$ | *Bacillus subtilis* group | Complete | Complete | Complete |
| $I_1$ | *Bacillus rubricus, B. rufus, or B. mycoides corallinus* | Complete | Complete | Complete |
| $I_2$ | | Complete | Complete | ¼ inch |
| $I_3$ | | None | None | None |
| $I_4$ | | None | None | None |
| $I_5$ | | None | None | None |
| $I_6$ | | None | None | None |
| $I_7$ | *Streptococcus lactis* | Complete | Complete | Complete |
| $I_8$ | *Bacillus brevis* or *B. subtilis* var. *globigii* | Complete | Complete | Complete |
| $I_9$ | | None | None | None |
| $I_{10}$ | Lactobacillus species | Complete | Complete | Complete |
| $I_{11}$ | | None | None | None |
| $I_{12}$ | *Bacterium linens* or Corynebacterium species | Complete | Complete | Complete |
| $I_{13}$ | | None | None | None |

After the final dentin fragment weighing, the broth was analyzed for total calcium using the following technic: Five cubic centimeters of the sample was used per 5 c.c. of trichloracetic acid and the protein material precipitated. Negligible calcium was believed to be in the precipitate, but since correlation between variants and series was the main goal this small amount was disregarded.

Five cubic centimeters of the filtrate was treated with 2 drops methyl red indicator and 1 c.c. of 4 per cent ammonium oxalate. Ammonium hydroxide was added from a dropper until the indicator just turned yellow, then 1.° N

TABLE II

CROSS ANTAGONISTIC ACTION AMONG ORGANISMS FROM SALIVA

| NO. | $S_2$ (IN.) | $S_7$ (IN.) | $S_9$ (IN.) | $S_{10}$ (IN.) | $S_{12}$ (IN.) | $S_{14}$ (IN.) | $S_{15}$ (IN.) | $I_1$ (IN.) | $I_2$ (IN.) | $I_7$ (IN.) | $I_8$ (IN.) | $I_{10}$ (IN.) | $I_{12}$ (IN.) | $S_{13}$ (IN.) | $S_{17}$ (IN.) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $S_2$ | | 0 | 0 | Comp. | 0 | 0 | 0 | 0 | 0 | 0 | ? | 0 | 0 | Comp. | 0 |
| | | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 1/32 | 0 | 0 | Comp. | 0 |
| $S_7$ | ? | | ? | Comp. | 0 | 0 | 0 | 1/2 | 1/8 | 0 | 0 | 0 | 0 | 1 1/2 | 0 |
| | 0 | | 1/8 | | 0 | 0 | 0 | 1/2 | 1/8 | 0 | 0 | 0 | 0 | 1 1/2 | 0 |
| $S_9$ | 0 | 0 | | Comp. | 0 | 2 | 0 | 0 | Comp. | 0 | 0 | 0 | 0 | Comp. | ? |
| | 0 | 0 | | | 0 | 2 | 0 | 0 | Poor growth | 0 | 0 | 0 | 0 | 1/4 | |
| $S_{10}$ | 0 | | | | | | | | | | | | | | |
| | 0 | | | | | | | | | | | | | | |
| $S_{12}$ | ? | 0 | ? | Comp. | | ? | 0 | Comp. | 3/8 | 0 | ? | ? | 1/8 | Comp. | 1/8 |
| | 1/8 | 0 | 1/4 | | | 0 | 0 | Comp. | 3/8 | 0 | ? | 0 | 1/8 | Poor growth | ? |
| $S_{14}$ | 0 | 0 | 0 | Comp. | 0 | | ? | 0 | 0 | 0 | 0 | 0 | 0 | 1/4 | 0 |
| | 0 | 0 | 0 | | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ? | 0 |
| $S_{15}$ | 0 | ? | 1/2 | Comp. | 1/4 | 1/4 | | Comp. | Comp. | 0 | 3/8 | 0 | 3/8 | 1 1/2 | 1/8 |
| | 0 | Slight | 1/2 | | 1/4 | 1/4 | | Comp. | Comp. | 0 | 0 | 0 | 1/16 | 1 | Slight |
| $I_1$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $I_2$ | 0 | 0 | 0 | Comp. | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 |
| $I_7$ | 0 | 0 | 1/4 | Comp. | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 1/32 |
| | 0 | 0 | 1/4 | | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 1/32 |
| $I_8$ | 3/16 | 1/8 | Comp. | Comp. | 1/4 | 1/4 | 1/4 | 1 | 3/8 | 0 | | 0 | 1/4 | 1/4 | 1/4 |
| | 1/8 | 1/8 | Comp. | Comp. | 1/4 | 1/4 | 1/4 | 1 | 3/8 | 0 | | 0 | 1/4 | 1/4 | 3/16 |
| $I_{10}$ | 0 | 0 | 0 | Comp. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 |
| | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 |
| $I_{12}$ | 0 | 0 | 0 | Comp. | 0 | 0 | 0 | 0 | 1/2 | 0 | 0 | 0 | | 0 | 0 |
| | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | | 0 | 0 |
| $S_{13}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Comp. | 0 | 0 | 0 | 0 | | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Comp. | 0 | 0 | 0 | 0 | | 0 |
| $S_{17}$ | 2 | 1 | 1 | 1 | 1/4 | Comp. | 1/8 | 2 | 2 | 0 | 1 | 0 | 3/4 | | |
| | 2 | 1 | 3/4 | 3/4 | 1/4 | Comp. | 1/8 | 2 | 2 | 0 | 1 | 0 | 3/4 | | |

Vertical headings on left indicate test organism.
Horizontal headings at top indicate organisms that were inhibited.
0 = no inhibition.
Comp. = complete inhibition.
$S_{17}$ = spreading growth.
$S_{10}$ = grows slowly.

hydrochloric acid added until a light pink color was obtained. The solutions were stirred with footed glass stirring rods which were removed later and hung on a numbered rack. After 1 hour the tubes were centrifuged, the supernatant liquid was poured off, the tubes inverted on paper or cloth to drain. A wash solution of alcohol and ether was added and the precipitate washed twice and recentrifuged each time. After the final washing the precipitate was dissolved in 4 c.c. of 1.° N perchloric acid with stirring rods as above and titration carried out in the centrifuge tube at room temperature with saponin indicator and ammonium hexanitratocerate as the oxidizing media. The amount of calcium was calculated as per cent loss or gain from the dentin fragments.

## RESULTS

The degree of antagonistic activity as measured by the streak-plate test is given in Table I. It can be seen that the various bacteria secured from saliva showed different degrees of effectiveness against the 3 lactobacillus strains, 15 of the 30 showed some antagonism. Although the *Bacillus brevis* and *subtilis* groups occur more frequently, there are several different species represented.

The antagonistic action of the organisms secured from saliva against each other is given in Table II. The degree of effectiveness varied considerably. This ranged from complete inability to inhibit any of the other organisms ($I_1$) to inhibition of 12 of 14 ($I_8$).

TABLE III

EFFECT OF ORGANISMS FROM SALIVA IN PREVENTING TOOTH FRAGMENT WEIGHT AND CALCIUM LOSS AND ACID PRODUCTION BY LACTOBACILLUS IN NUTRIENT BROTH AND 5 PER CENT SUCROSE

| | INCUBATION TIME | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4 HOURS | | | 24 HOURS | | | 48 HOURS | | | 72 HOURS | | |
| NUMBER | % WT. + − | % CA. + − | pH | % WT. + − | % CA. + − | pH | % WT. + − | % CA. + − | pH | % WT. + − | % CA. + − | pH |
| $S_2$ | +0.12 | −0.04 | 5.6 | −0.86 | −0.22 | 5.2 | −2.4 | −0.82 | 4.8 | −4.4 | −1.7 | 4.7 |
| $S_7$ | +0.18 | −0.08 | 6.4 | −0.87 | −0.42 | 5.1 | −4.9 | −2.0 | 4.6 | −7.3 | −2.7 | 4.6 |
| $S_9$ | +0.10 | −0.08 | 5.4 | −0.61 | −0.51 | 4.7 | −2.0 | −0.90 | 4.7 | −4.0 | −1.7 | 4.5 |
| $S_{12}$ | +0.11 | 0.00 | 5.8 | −0.12 | −0.18 | 5.1 | −1.2 | −0.56 | 5.2 | −5.6 | −2.67 | 4.7 |
| $S_{13}$ | +0.41 | −0.05 | 5.7 | −0.25 | −0.25 | 5.3 | −1.1 | −0.81 | 4.9 | −3.5 | −1.5 | 4.6 |
| $S_{14}$ | −0.12 | −0.04 | 6.1 | +0.02 | −0.07 | 5.6 | −1.0 | −0.56 | 5.1 | −3.5 | −1.2 | 4.7 |
| $S_{15}$ | +0.50 | −0.05 | 5.7 | −0.61 | −0.27 | 5.4 | −2.0 | −1.07 | 5.1 | −4.8 | −1.99 | 4.9 |
| $S_{17}$ | −0.41 | −0.08 | 5.3 | +0.22 | −0.05 | 5.5 | −0.42 | −0.31 | 5.4 | −3.2 | −1.2 | 5.0 |
| $I_1$ | −0.02 | −0.14 | 5.4 | 0.03 | 0.30 | 5.4 | 2.0 | 0.94 | 4.9 | 3.5 | 1.5 | 4.9 |
| $I_2$ | +0.20 | −0.04 | 6.4 | +0.08 | −0.14 | 6.4 | +0.52 | −0.08 | 6.6 | +0.48 | −0.06 | 7.1 |
| $I_7$ | +0.10 | −0.03 | 5.6 | −1.5 | −0.62 | 5.0 | −2.8 | −1.13 | 4.6 | −3.5 | −1.64 | 4.7 |
| $I_8$ | −0.12 | −0.04 | 6.1 | −0.42 | −0.10 | 5.8 | −0.65 | −0.64 | 5.0 | −4.8 | −1.8 | 4.9 |
| $I_{10}$ | −0.43 | −0.11 | 5.4 | −2.0 | −1.12 | 4.5 | −6.0 | −2.4 | 4.5 | −7.0 | −3.3 | 4.6 |
| $I_{12}$ | −0.12 | −0.05 | 6.0 | −1.4 | −0.51 | 4.9 | −2.2 | −0.07 | 4.9 | −4.3 | −2.19 | 4.9 |
| Control | −0.10 | −0.06 | 6.1 | −0.44 | −0.28 | 4.9 | −2.1 | −0.93 | 4.7 | −2.5 | −12.0 | 4.8 |

Table III shows the results of the ability of the antagonistic bacteria in preventing weight and calcium loss from dentin fragments. It was found that in only two instances ($S_{12}$ and $I_2$) was there any appreciable difference from the control.

## DISCUSSION AND SUMMARY

It is felt this investigation has shown that in saliva pools, on the basis of the agar streak-plate test, there are bacteria present which are antagonistic to three strains of lactobacilli. These bacteria in many instances can be antagonistic to each other. However, in broth cultures with sucrose, where growth conditions were favorable for lactobacillus growth, these antagonistic organisms usually were ineffective in preventing acidogenesis as measured by dentin fragment weight, calcium loss, and pH decrease.

It is recognized that these organisms may be transient inhabitants of the oral cavity, and the apparent antagonistic effect may be simply an alteration of the culture media by depletion of some essential metabolic requirement or pH change. These and other possibilities are being investigated.

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# INDUCED ALTERATIONS IN THE REACTION CAPACITY OF STREPTOCOCCUS ODONTOLYTICUS

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THAT bacteria vary in form, in function, and in chemical and antigenic composition is generally accepted. Zinsser and Bayne-Jones[3] observe: "So many instances of bacterial variation within the so-called 'species' have been rigorously demonstrated and readily confirmed that the fact of bacterial variability is no longer in question. These variations should now become a part of the description of the organism."

We have previously reported on a mucoid variant of *Streptococcus salivarius* (*Str. odontolyticus*) which is associated with acute dental caries and requires sucrose for the maintenance of the mucoid phase.[1, 2] The following report is a continuation of these studies.

*Experiment 1.*—Saliva from a caries-susceptible individual was streaked on nutrient agar (Difco dehydrated—final pH 7.3) to which 2 per cent human blood and 2 per cent sucrose has been added. The resultant growth is shown in Fig. 1. An isolated mucoid colony was transferred by platinum loop to a test tube containing autoclaved skimmed milk. Coagulation occurred in fourteen hours. In forty-eight hours the culture was replated on nutrient blood agar containing 2 per cent sucrose and incubated for forty-eight hours. A mucoid colony was again transferred by platinum loop to milk as before. This procedure was repeated at regular intervals for five months with no other growth occurring than that which had been selected from the original saliva plate.

On November 24, 1949, tubes containing 10 c.c. of skimmed milk and 1 Gm. of $CaCo_3$ were autoclaved and inoculated with *Str. odontolyticus* and incubated for twenty-four hours. The pH dropped to 4.2 with resultant coagulation of the milk. Thereafter, tubes were regularly agitated to keep the curd broken.* Resting samples (taken prior to agitation) showed a gradual shift in pH, and on the tenth day the pH was six. After the first twenty-four hours incubation of the inoculated $CaCo_3$ tubes, it was obvious that digestion of the milk was taking place. At the expiration of two weeks this digestion had progressed to a marked degree.

*Experiment 2.*—A tooth which had not been exposed to oral fluids was placed in milk $CaCo_3$ and inoculated with *Str. odontolyticus* and incubated for three weeks. The tube was agitated as before. At this time there was some superficial decalcification of the enamel; however, the most striking phenomenon was the penetration which was occurring between enamel and dentin.

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*If tubes are agitated at an early date and the shaking repeated sufficiently to prevent curding of the milk, then little or no digestion takes place.

At this time the tooth was resterilized by moist heat 160° F. for six hours and incubated as previously for three additional weeks. On re-examination, the spreading was pronounced as shown in Fig. 2. The exposed dentin had a light golden-yellow pigmentation and had the gross appearance of a superficial digestion having taken place.

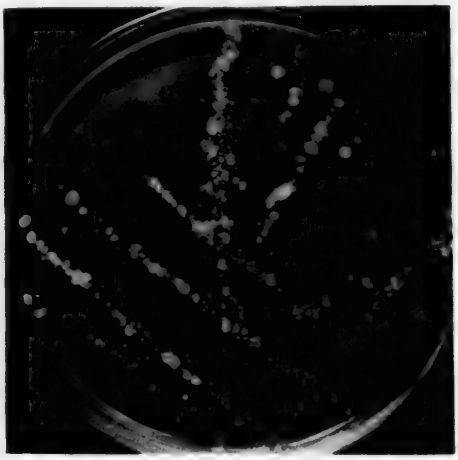

Fig. 1.—Resultant growth procured after streaking saliva of a caries-susceptible individual on nutrient blood agar containing 2 per cent sucrose. Note hemispherical mucoid colonies.

The destruction which has taken place in this tooth is not typical of the generally accepted picture of the carious lesion; however, it may illustrate one of the phases associated with typical dental caries.

*Experiment 3.*—Test tubes containing potato and distilled water, also potato, distilled water, and $CaCo_3$ were inoculated with *Str. odontolyticus* derived from milk-$CaCo_3$ tube. These tubes were incubated for five days.

Fig. 2.—Note penetration between enamel and dentin.

In the case of the plain potato tubes, a profuse growth was present. The pH was 4.2 and the organism grew in long chains. In the case of the tubes containing potato and $CaCo_3$, the pH was 7.5 and the growth was scanty even on microscopic examination, and the organism bore more resemblance to enterococci than to the original streptococci. At this time the organism had lost its

mucoid characteristics, growing on nutrient blood agar plus 2 per cent sucrose as small glasslike colonies which were of firm consistency and adherent to the medium.

## SUMMARY

The *Str. odontolyticus* has been demonstrated to digest milk under certain in vitro conditions. An extracted tooth placed in milk-$CaCO_3$ and inoculated with *Str. odontolyticus* evidenced superficial enamel decalcification and a penetration and spreading of a destructive process between enamel and dentin. In alkaline media (potato and $CaCo_3$), the organism lost its mucoid characteristics.

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# EFFECT OF A SELECTED NITROFURAN, FURACIN, ON THE ORAL LACTOBACILLUS COUNT

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PRELIMINARY data[1] concerning the action of Furacin (2 nitro-5-furaldehyde semicarboazone) and related nitrofurans on various members of the oral flora indicated that the lactobacilli were not particularly sensitive to their action. Only 9 of the 18 strains tested were inhibited by the most active of these compounds prepared as 1:500 and 1:1000 (W/V) concentrations in propylene glycol. It should follow that local application, even if no other factors were involved, would not markedly influence the number of these organisms in the oral cavity as reflected by the lactobacillus count. This was determined by trial for one nitrofuran, Furacin,* incorporated in sugar-coated gum tablets. It was thought of interest to report the results of this work as another example of evaluation of an agent of known chemotherapeutic value[2, 3] in the possible control of caries activity. Similar studies with various chemicals and antibiotics have been reported, among which may be listed those of Stefan and Miller[4] with urea and Zephiran chloride, Kesel[5] with urea and dibasic ammonium phosphate, Hill and Kniesner[6] with penicillin, and Rapp, Gurney, Olen, Shafer, and Hein[7] with chlorophyll.

When this study began (1948) there was no published literature concerning the action of nitrofurans on the oral flora. Recently, Dreizen, Greene, and Spies[8, 9] have reported on the in vivo and in vitro action of various nitrofurans on acid production by microorganisms in culture media and saliva. They found that initial concentrations of 1.0 to 3.0 mg./10 ml. medium of the 10 nitrofurans tested inhibited completely for 24 hours' growth and acid production by *Lactobacillus acidophilus* (Hadley); this effect lasted 72 hours for 5 of the 10 compounds but only when the concentrations were increased up to 2.0 to 4.0 mg./10 ml. medium. The bacteriostatic activity of the remaining 5 nitrofurans was lost after 24 hours. This delay in the growth curve of *L. acidophilus* (Hadley) was similar to the prolongation by nitrofurans of the "lag" period of *Staphylococcus aureus* as reported by Cramer and Dodd.[10] On the other hand, 500 to 1000 $\mu$g of these agents added to 10 ml. aliquots of saliva prevented acid production in a high percentage of specimens. It seems rather curious, however, that these workers labeled data obtained with stimulated specimens of saliva, collected from patients chewing paraffin tablets containing the drug, as in vivo effects of nitrofurans. In our study the nitrofuran was at least allowed to act in the mouth for some time and, subsequently, the lactobacillus counts were determined in the presumable absence of the nitrofuran in the specimens of saliva.

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## PROCEDURE

To test the effect of Furacin on the oral lactobacilli, it was necessary to select individuals with counts sufficiently high to give some indication of consistent quantitative change. A class of 75 students was screened (1948) for desirable subjects but only 19 were found to have counts higher than 5,000 lactobacilli/ml. saliva. All agreed to serve as an experimental group and were studied in a manner to be described. It was recognized that the number of cases was too small to have any real significance; consequently, the experiment was repeated for reproducibility with a second group of 19 cases similarly selected (1949) from another class.

The treatment of each group was identical: first, a preliminary period of 10 days during which no gum was chewed; second, a trial period of 10 days during which sugar-coated gum tablets were chewed for 1 hour 3 times daily (between meals and after dinner), and third, a test period of 10 days during which tablets of sugar-coated gum containing 100 mg. of Furacin/tablet were chewed with the same frequency and duration as the drugless gum. Specimens of saliva were collected before breakfast on every third day of each period. The lactobacillus counts were made by the method of Hadley.[11]

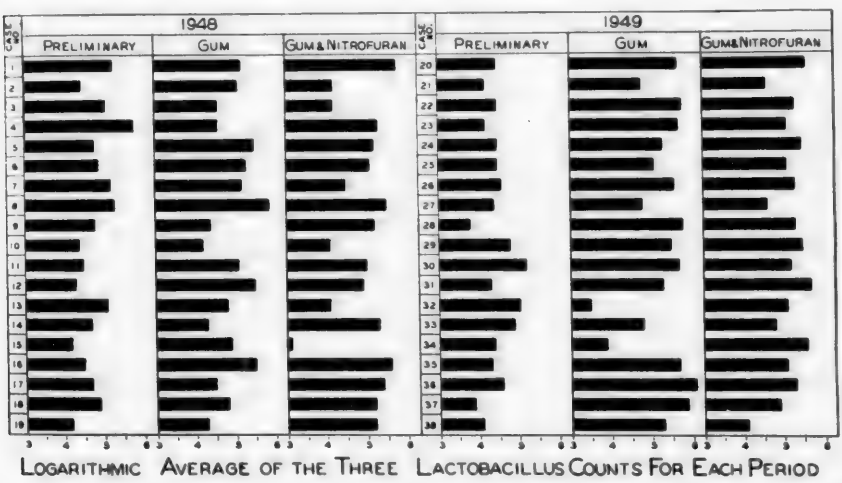


Fig. 1.—Response of individual lactobacillus counts during a period of 10 days chewing sugar-coated gum with and without Furacin compared to those in a 10-day period without the gum.

## RESULTS

The results obtained in this evaluation of the effect of Furacin on the oral lactobacillus count are best illustrated graphically. The average, expressed in logarithms, of the 3 lactobacillus counts taken during each period is shown for the individuals of both groups in Fig. 1.

It is to be noted in Fig. 1 that the lactobacillus count was definitely increased in most of the subjects following the introduction of the sugar-coated gum. This count, which is widely accepted as an index of caries susceptibility,

dropped only slightly with the use of Furacin, and in the majority of individuals did not return to the levels established in the preliminary or reference period; this negative response was also slightly more pronounced in the second than the first group. These trends were clear when the individual counts were averaged for each group. The results of this compilation are presented in Fig. 2.

There are three features to be gathered from Fig. 2. First, the response of the 2 groups to the treatment was essentially similar; second, the lactobacillus count was increased considerably following the introduction of the sugar-coated gum; and, third, Furacin did not reduce this elevated lactobacillus count to any apparent degree. There was a much greater difference in the average lactobacillus counts of the second group (1949) than the first group (1948) in response to the sugar-coated gum, but this did not seem to have any particular influence on the end result when subjected to the action of Furacin. On the other hand, the conclusion that 300 mg./day of Furacin incorporated in sugar-coated gum did not significantly reduce the lactobacillus count was substantiated by the practical duplication of results from both groups.

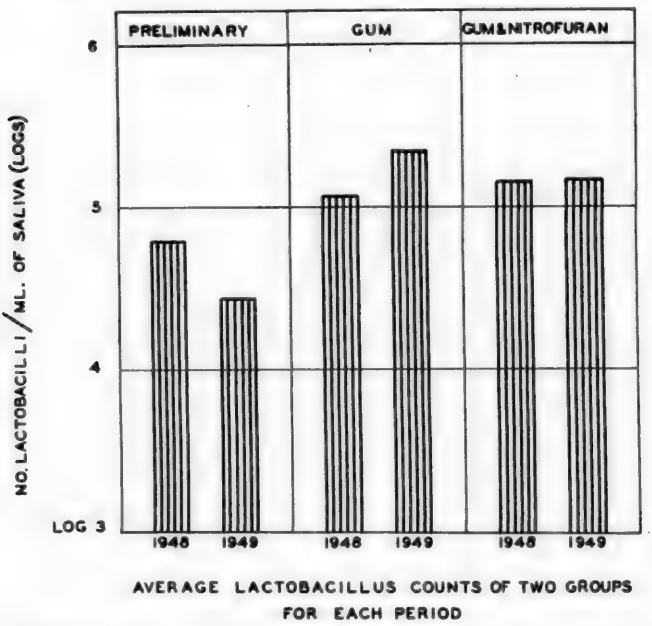


Fig. 2.—Response of the group lactobacillus count during periods of 10 days of chewing sugar-coated gum tablets with and without Furacin compared to those in a 10-day period without gum.

## DISCUSSION

The problem of chemotherapy for the prevention of dental caries is most difficult. It is almost impossible to retain adequate chemotherapeutic levels by frequent topical application of the drugs in the oral cavity over a sufficient length of time to accomplish the results desired. This can be exemplified by the

relatively low reduction in both caries incidence and lactobacillus counts attributable to the action of penicillin in the clinical study of Hill and Kniesner,[6] although they pointed out the well-known fact that humans are the poorest of experimental animals on the basis of reliability. The same point applies to this study.

In view of the slight to moderate susceptibility of the oral lactobacillus to Furacin and related nitrofurans, it is small wonder that there was apparent failure in the present experiment. Whether the desired inhibitory effect could be obtained over a longer period, which would also permit clinical and laboratory correlation, is a matter to be settled only by trial. However, such a study would be hampered by the extreme bitterness of the drug and its tendency to produce nausea when slowly swallowed. It appears unlikely that this nitrofuran will be employed in the chemotherapy of dental caries.

## SUMMARY

The consumption of 300 mg./day of Furacin incorporated in sugar-coated gum tablets did not reduce in any significant way the oral lactobacillus count definitely increased by the sugar-coated gum alone.

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# STUDIES ON EFFECT OF GONADECTOMY ON DENTAL CARIES ACTIVITY IN WHITE RATS AND IN COTTON RATS

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KEYES[1] has studied the effect of gonadectomy on the initiation and development of carious lesions in the Syrian hamster. Castration of weanling male hamsters was found to result in a significant reduction in dental caries incidence. Ovariectomy of weanling female hamsters tended to reduce the dental caries activity but not to a statistically significant degree. However, Hodge[2] was unable to detect any difference in dental caries experience between normal and ovariectomized albino rats which were maintained on a coarse particle caries-producing diet for 19 months; in this case, ovariectomy and the beginning of the experiment for both groups did not occur until the rats were 12 months of age. In the present study, cotton rats and white rats of caries-susceptible strains were subjected to gonadectomy at weaning to determine the effect upon dental caries activity in animals maintained on a caries-producing purified diet during the experimental period.

## EXPERIMENTAL

The subjects in the first experiment were 152 weanling albino rats (*Mus norvegicus*), representing 30 litters born to females which had been maintained throughout their reproductive lives on purified ration 100,[3] a procedure which has previously been shown to result in offspring of high caries susceptibility.[4] Forty-four males and 30 females were maintained as controls; the testes of 46 males and the ovaries of 32 females were removed while the animals were under ether anesthesia. The normal and gonadectomized rats were maintained in groups of 3 or 4 of the same sex in large screen-bottom cages and were offered ration 100 and water ad libitum for the next 20 weeks.

The subjects in the second experiment were 38 weanling cotton rats (*Sigmondon hispidus hispidus*), representing 9 litters born to females in our caries-susceptible colony. Eight males and 9 females were maintained as controls; the testes of 9 males and the ovaries of 12 females were surgically removed. The control and experimental cotton rats were maintained for the following 14 weeks in individual screen-bottom cages and were offered ration 100 and water ad libitum.

At the end of the respective experimental periods, the animals were sacrificed and their heads fixed in 95 per cent ethanol. After 48 hours or more, the heads were skinned and the molar teeth examined to determine the number and size of the carious lesions.

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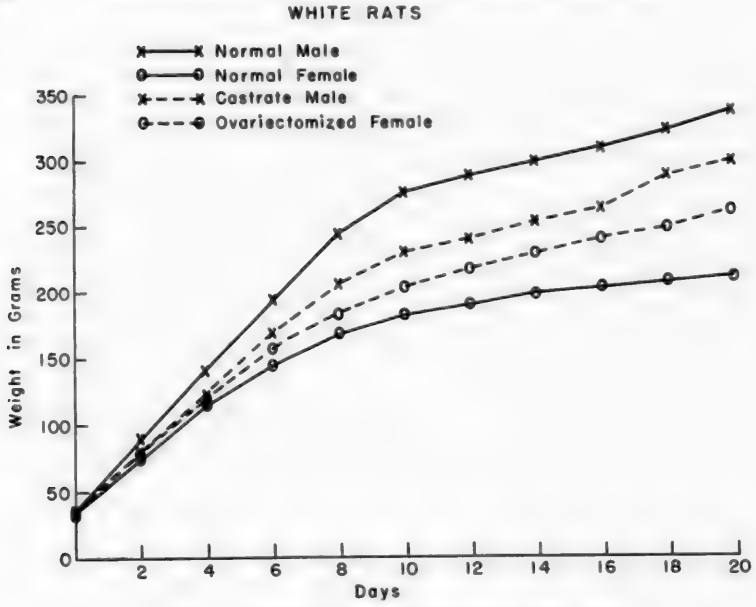


Fig. 1.—Average rates of growth for albino rats.

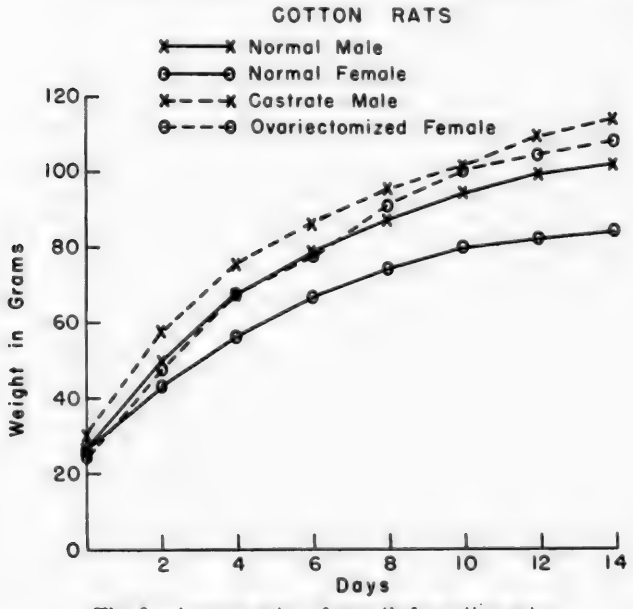


Fig. 2.—Average rates of growth for cotton rats.

## RESULTS

The average rates of growth for the albino rats are presented in Fig. 1 and for the cotton rats in Fig. 2. The intact male albino rats grew at an appreciably faster average rate and attained a greater average weight than did

their castrated littermates. The ovariectomized female albino rats grew considerably faster and attained a greater body weight than their intact female littermates. The intact male, the castrated male and ovariectomized female cotton rats grew at approximately the same rates and had reached about the same average weights at the termination of the experiment. All 3 groups considerably exceeded the growth performance of the intact female cotton rats.

The data on the incidence and extent of carious lesions in the various groups of rodents in the first and second experiments are presented in Tables I and II, respectively. In both species, no difference in the average dental caries incidence was observed between intact males and females. This observation is further substantiated by unpublished data from much larger groups of animals.

TABLE I

EFFECT OF GONADECTOMY ON DEVELOPMENT OF CARIOUS LESIONS IN THE WHITE RAT

| CONDITION | NUMBER OF RATS | NUMBER OF CARIOUS MOLARS | | | NUMBER OF CARIOUS LESIONS | | | EXTENT OF CARIOUS LESIONS | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | AVG. | S.E.M.* | C.R.† | AVG. | S.E.M.* | C.R.† | AVG. | S.E.M.* | C.R.† |
| Normal male | 44 | 7.5 | 0.5 | | 12.4 | 1.0 | | 32+ | 4+ | |
| | | | | 2.9 | | | 3.5 | | | 3.3 |
| Castrated male | 46 | 5.5 | 0.5 | | 7.9 | 0.8 | | 17+ | 2+ | |
| Normal female | 30 | 7.1 | 0.5 | | 11.9 | 1.1 | | 31+ | 4+ | |
| | | | | 2.0 | | | 2.0 | | | 1.8 |
| Ovariectomized female | 32 | 5.4 | 0.7 | | 8.5 | 1.3 | | 21+ | 4+ | |

*Standard error of mean.

†Critical ratio—the ratio of the difference between two means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when the critical ratio is between 2.0 and 2.9, the difference is of borderline significance; when the critical ratio is 3.0 or higher, the difference is highly significant. The crisscross lines in the above table connect sets of data which are being compared for the critical ratio test of significance.

In both species, castration and ovariectomy resulted in slight to marked reductions in the average number of carious molars, the average number of carious lesions, and the average extent of carious lesions. Castration of male albino rats resulted in highly significant reductions in the average number of carious lesions and the average extent of carious lesions but the reduction was only of borderline statistical significance in the case of the average number of carious molars. Ovariectomy of albino rats resulted in reductions of borderline significance for the average number of carious molars and the average number of carious lesions but the slight reduction for the average extent of carious lesions was statistically insignificant.

Castration of male cotton rats was found to cause a reduction of borderline significance for the average number of carious molars but the small reductions in the average number of carious lesions and the average extent of carious lesions were found to be statistically insignificant. Following ovariectomy of cotton rats, the resulting slight reductions in the three categories of dental caries experience used herein for measurement of dental caries involvement were found to be insignificant by the critical ratio test for statistical significance.

It is of interest to call attention to the fact that in every case the difference between intact and gonadectomized rodents tended toward a lower dental caries experience among the gonadectomized animals. Even though these reductions were statistically insignificant in all three categories for female cotton rats and in some categories for male cotton rats and female albino rats, the general trend toward a reduction strongly suggests some systemic influence upon dental caries activity mediated through the gonads. Furthermore, in both sexes of the two species studied in the present investigation, the percentage decreases in dental caries activity among the gonadectomized animals were very similar to those described by Keyes[1] in the Syrian hamster. This similarity occurred despite the fact that the composition of the diets in the two investigations differed considerably.

TABLE II

EFFECT OF GONADECTOMY ON DEVELOPMENT OF CARIOUS LESIONS IN THE COTTON RAT

| CONDITION | NUMBER OF RATS | NUMBER OF CARIOUS MOLARS | | | NUMBER OF CARIOUS LESIONS | | | EXTENT OF CARIOUS LESIONS | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | AVG. | S.E.M.* | C.R.† | AVG. | S.E.M.* | C.R.† | AVG. | S.E.M.* | C.R.† |
| Normal male | 8 | 11.3 | 0.1 | 2.9 | 28.8 | 1.5 | 1.6 | 91+ | 9+ | 1.9 |
| Castrated male | 9 | 10.9 | 0.1 | | 25.3 | 1.7 | | 68+ | 8+ | |
| Normal female | 9 | 11.3 | 0.1 | 0.9 | 26.8 | 1.3 | 0.8 | 75+ | 7+ | 1.2 |
| Ovariectomized female | 12 | 11.1 | 0.2 | | 25.3 | 1.5 | | 62+ | 8+ | |

*Standard error of mean.

†Critical ratio—the ratio of the difference between two means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when the critical ratio is between 2.0 and 2.9, the difference is of borderline significance; when the critical ratio is 3.0 or higher, the difference is highly significant. The crisscross lines in the above table connect sets of data which are being compared for the critical ratio test of significance.

## SUMMARY

The influence of gonadectomy upon the dental caries activity has been studied in caries-susceptible white rats and cotton rats which were fed a highly purified caries-producing diet.

In both species, there was a consistent tendency toward a lesser average incidence of tooth decay in the gonadectomized rodents than in the intact littermates. The reduction in dental caries incidence was appreciably higher among orchidectomized males than among ovariectomized females. No difference was observed between the average dental caries activity in males and females for each species.

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# AN EFFECT OF REPRODUCTION ON THE DENTAL CARIES ACTIVITY IN WHITE RATS

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THE old adage "a tooth for every child" is still often heard quoted with emphasis among practicing dentists and laymen. Numerous clinical surveys have been reported in which the relation of pregnancy, lactation, number of pregnancies, and the patient's age to the current dental caries activity, or to the lifetime dental caries experience, were explored. The results of these surveys varied greatly. From Switzerland, Gerson[1] reported a higher incidence of superficial caries, of deep carious lesions, and of extractions during pregnancy than in nonpregnant women. Biro,[2] Starobinsky,[3] Day and Shourie,[4] and others observed no difference between the dental caries activity in nulliparous women and pregnant women within the same age groups. In contrast, Ziskin[5] and Ziskin and Hotelling[6] believed that there was a lower incidence of tooth decay among women who had had one or more children than among those who had had none. Despite this range of disagreement, laboratory studies of any possible relationships of pregnancy and lactation to the susceptibility to tooth decay have been infrequent. Toverud[7] observed that the incisors of control female rats which had been maintained on a partially calcium-deficient diet were essentially normal, whereas the incisors of female rats fed the same diet during one or two pregnancies contained less ash, calcium, and phosphorus. Deakins,[8] and Deakins and Looby[9] concluded from specific gravity studies of dentin from carious teeth that no demineralization of sound dentin occurred during pregnancy and that neither gestation nor demineralization of the teeth was associated with the susceptibility to tooth decay. Dragiff and Karshan[10] observed no differences between the ash, calcium, or phosphorus concentrations of the root dentin from pregnant and that from nonpregnant women. However, no studies are known to have been conducted to determine whether or not pregnancy and lactation altered the initiation and development of carious lesions in caries-susceptible animals. In the present study, caries-susceptible rats were maintained throughout pregnancy and lactation on a caries-producing diet known to be adequate in all nutrients which have been shown to be essential for the normal growth and development of the rat.

## EXPERIMENTAL

The subjects in this investigation were 30 pairs of weanling, female albino rats *(Mus norvegicus)* from 30 litters born to females which had been maintained throughout their reproductive lives on ration 100,[11] a procedure which has been

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shown to result in offspring with a high susceptibility to tooth decay.[12] Each of the 60 weanling, female rats in the present experiment was offered ration 100 and water ad libitum throughout the entire experiment. When the rats were sexually mature, one member of each pair was bred to a closely related male while the other representative of the pair was maintained as an unmated control. The control females also served as the controls in an experiment conducted simultaneously to study the effect of gonadectomy on dental caries activity.[13] Throughout this study, the control females were maintained in cages with screen floors. The representatives of the experimental group were kept for the few days prior to and after parturition in individual cages with shavings as bedding. As soon as the offspring were sufficiently large, the families were transferred to cages with screen floors.

In order to produce the maximum strain on the maternal organism, litter size was not reduced regardless of the number of offspring. The litters were weaned at an average age of 25 days and at an average weight of 52 grams. Since 8 of the females in the experimental group completed one reproductive cycle several weeks prior to the proposed termination of the investigation at 20 weeks, they were mated again 10 to 14 days after the first litter was weaned. Each of these 8 females bore a second litter between the nineteenth and twenty-second experimental weeks. When the members of these litters were weaned at 25 days of age, the mothers were sacrificed. The remaining 22 females in the experimental group, and the 30 females in the control group, were sacrificed at the end of the twentieth experimental week. The heads were preserved in 95 per cent ethanol and then evaluated for number and extent of carious lesions by examination under a dissecting microscope and by grinding successive planes parallel to the occlusal surface.

## RESULTS

The average number of carious molars, the average number of carious lesions, and the average extent of carious lesions are presented in Table I. Since the dental caries activity observed in the 8 females which had had 2 litters fell entirely within the range of values for the 22 females which had had only 1 litter and a slightly shorter study period, the values for all 30 experimental females were averaged together. In each of the 3 categories for measuring dental caries experience, the average figures for the females in the experimental group were lower than for the control group. Upon statistical evaluation by the critical ratio test, the decrease in the average number of carious molars and the decrease in the average number of carious lesions observed among the females which had completed 1 or 2 reproductive cycles were judged to be borderline in statistical significance. However, the lower average extent of carious lesions among the experimental females than among the littermate controls was considered to be highly significant.

In comparison with the caries incidence of their ovariectomized sisters reported in the previous paper,[13] the mated females in this experiment had almost identical experiences in all 3 categories.

In the course of the 38 pregnancies, 264 babies were born and 232 weaned. These values represented an average of 7.0 babies (range 1 to 10) born per litter and an average of 6.1 (range 0 to 10) weaned per litter. Examination of the dental caries data failed to reveal any correlation between litter size and dental caries incidence.

TABLE I

EFFECT OF PREGNANCY AND LACTATION ON DENTAL CARIES ACTIVITY IN WHITE RATS

| NUMBER OF COMPLETED REPRODUCTIVE CYCLES | NUMBER OF RATS | NUMBER OF CARIOUS MOLARS | | | NUMBER OF CARIOUS LESIONS | | | EXTENT OF CARIOUS LESIONS | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | AVG. | S.E.M.* | C.R.† | AVG. | S.E.M.* | C.R.† | AVG. | S.E.M.* | C.R.† |
| 0 | 30 | 7.1 | 0.5 | 2.4 | 11.9 | 1.1 | 2.8 | 31+ | 4+ | 3.0 |
| 1 or 2 | 30 | 5.2 | 0.6 | | 7.6 | 1.1 | | 16+ | 3+ | |

*Standard error of mean

†Critical ratio—the ratio of the difference between 2 means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when the critical ratio is between 2.0 and 2.9, the difference is of borderline significance; when the critical ratio is 3.0 or higher, the difference is highly significant. The crisscross lines in the above table connect sets of data which are being compared for the critical ratio test of significance.

## DISCUSSION

The rats in this experiment received a diet which, on the basis of present knowledge, is believed to contain abundant amounts of all nutrients known to be essential for the growth and maintenance of this species. Under these experimental circumstances, the dental caries incidence of female rats subjected to the strain of 1 or 2 complete reproductive cycles was, if anything, reduced below that of their control, unmated sisters, and was as low as that of their ovariectomized sisters reported in the previous paper.[13] The importance of this reported reduction and the mechanism through which such a decrease in dental caries development might be mediated is unknown. Further experiments under a variety of carefully controlled conditions need to be conducted in order to explore the various ramifications of this problem. For example, it is impossible to predict from this study whether or not various borderline deficiencies of essential nutrients imposed during reproduction might influence the dental caries incidence of the reproducing females.

The various clinical studies on the possible relationship of reproduction to the dental caries activity of women have taken essentially no cognizance of the diets consumed by their control and experimental groups of women. Since these clinical studies were conducted in widely separated areas of the world, there is a definite possibility that the dietary pattern of the Swiss women studied by Gerson,[1] who reported a higher dental caries activity among the pregnant women, differed vastly from that of the American women studied by Ziskin[5] and Ziskin and Hotelling[6] who reported a slightly lower incidence of tooth decay among women with children.

## SUMMARY

The influence of reproduction upon dental caries experience has been investigated in caries-susceptible white rats which were maintained on a highly purified caries-producing diet.

Under the conditions of this experimental regimen, the 30 females which had completed one or two reproductive cycles had a slightly lower average number of carious molars, a slightly lower average number of carious lesions, and a significantly lower average extent of tooth decay than their litter mates which had never been pregnant.

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# THE EFFECT OF CONTROLLED WATER CONSUMPTION ON DENTAL CARIES IN WHITE RATS

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IN A previous study[1] no effect on dental caries was observed in white rats from a caries-susceptible strain on a coarse corn cariogenic dietary, drinking varying amounts of water ad libitum. These conclusions were reached by indirect means, using biostatistical methods. Other investigators have reported considerable reduction of dental caries in cotton rats fed whole milk dietaries[2, 3, 4] and moistened cariogenic rations.[4] In a study on 750 boys, two or three years after the boys ingested a pint of raw milk daily, "it was noticed that once they had had dental treatment, there was subsequently a remarkable diminution, almost to absence, of 'return' work for dental caries."[5]

Although these reports[2, 3, 4, 5] do not follow an experimental design identical to that to be reported here, they were the only reports available here[6] which seemed to have some relation to this study.

In order to check the results of the former analysis,[1] it was planned to analyze the direct effect of regulated water consumption on the dental caries incidence in white rats.

## PROCEDURE

Sixty weanling white rats, representing 17 litters from a caries-susceptible strain, were divided into 6 groups of 10 each, containing 5 males and 5 females in each group. The group received and consumed 20.0 c.c., 22.5 c.c., 25.0 c.c., 27.5 c.c., 30.0 c.c., and 32.5 c.c., respectively, of distilled drinking water daily. All rodents were fed the coarse corn cariogenic dietary used in other experiments.[1] The rodents were kept in a vermin-free, temperature-controlled room in cages constructed with wire bottoms and suspended from the shavings receiving their excreta. At the end of 100 days on this regimen, the animals were sacrificed and their teeth ground, examined, and dental caries recorded according to a standard procedure.[7] The technic of analysis of variance as described in a previous report[8] was applied to the results.

## RESULTS

As may be seen in Table I, the mean caries incidence for each group of rodents, consuming the 5 different quantities of distilled water, was between 13.9 and 17.4. The analysis of variance applied to the results revealed that there was no significant difference in the caries incidence between the groups consuming different quantities of distilled water over the 100-day period.

The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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TABLE I

DENTAL CARIES INCIDENCE IN WHITE RATS ON A COARSE CORN CARIOGENIC DIET AND REGULATED DISTILLED WATER CONSUMPTION FOR 100 DAYS

(Experiment Begun in Weanlings, 3 Weeks of Age)

| 20 c.c./day | | | 22.5 c.c./day | | | 25 c.c./day | | | 27.5 c.c./day | | | 30 c.c./day | | | 32.5 c.c./day | | | TOTAL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RAT NO. | SEX | CARIES INCIDENCE | RAT NO. | SEX | CARIES INCIDENCE | RAT NO. | SEX | CARIES INCIDENCE | RAT NO. | SEX | CARIES INCIDENCE | RAT NO. | SEX | CARIES INCIDENCE | RAT NO. | SEX | CARIES INCIDENCE | CARIES INCIDENCE |
| 443 | M | 21 | 444 | M | 15 | 445 | M | 17 | 446 | M | 11 | 447 | M | 20 | 448 | M | 25 | 109 |
| 437 | M | 22 | 438 | M | 11 | 439 | M | 18 | 440 | M | 18 | 441 | M | 20 | 424 | M | 19 | 108 |
| 431 | M | 15 | 432 | M | 12 | 433 | M | 21 | 434 | M | 19 | 423 | M | 21 | 430 | M | 17 | 105 |
| 419 | M | 16 | 420 | M | 16 | 421 | M | 24 | 422 | M | 11 | 429 | M | 21 | 436 | M | 15 | 103 |
| 425 | M | 6 | 426 | M | 11 | 427 | M | 6 | 428 | M | 15 | 435 | M | 13 | 442 | M | 20 | 71 |
| 455 | F | 16 | 456 | F | 15 | 457 | F | 16 | 458 | F | 8 | 477 | F | 19 | 460 | F | 20 | 94 |
| 461 | F | 19 | 462 | F | 22 | 463 | F | 20 | 464 | F | 12 | 459 | F | 18 | 472 | F | 6 | 97 |
| 467 | F | 20 | 468 | F | 17 | 469 | F | 20 | 470 | F | 14 | 453 | F | 15 | 478 | F | 14 | 100 |
| 473 | F | 20 | 474 | F | 12 | 475 | F | 17 | 476 | F | 15 | 465 | F | 13 | 454 | F | 23 | 100 |
| 449 | F | 15 | 450 | F | 16 | 451 | F | 12 | 452 | F | 16 | 471 | F | 13 | 466 | F | 15 | 87 |
| 10 | | 170 | 10 | | 147 | 10 | | 171 | 10 | | 139 | 10 | | 173 | 10 | | 174 | 974 |
| Mean | | 17.0 | | | 14.7 | | | 17.1 | | | 13.9 | | | 17.3 | | | 17.4 | 16.2 |

ANALYSIS OF VARIANCE

| SOURCE | SUM OF SQUARES | DEGREE OF FREEDOM | MEAN SQUARE | RATIO | F AT 0.01 |
|---|---|---|---|---|---|
| Treatment | 116.33 | 5 | 23.27 | 1.24 | 3.38 |
| Sex | 5.40 | 1 | 5.40 | 3.47 | 6302 |
| Error | 993.00 | 53 | 18.74 | | |
| Total | 1114.73 | 59 | | | |

No effect of water consumption or sex on dental caries incidence.

## DISCUSSION

The results of this study do not preclude the possibility that moist cariogenic dietaries of the coarse corn variety or fluid dietaries may influence dental caries incidence as has been reported [2, 3, 4, 5] due to the difference in experimental methods of procedure. It is obvious that varying water consumption at the time of comminution of a dry coarse corn cariogenic dietary may not bring into play the same factors which may be operating, as when the dietary itself is moistened prior to comminution or when a dietary is mostly fluid. This study does seem to support previous indirect observations which indicated that the quantity of distilled water intake per se is not an influencing factor in dental caries incidence in the rodents being studied here.

## CONCLUSIONS

1. In the group of rodents studied here, quantity of distilled water consumption appeared to have no effect on dental caries incidence.

2. This direct study seems to confirm what has been observed when using indirect means of evaluating caries incidence in another group of rodents from the same strain who drank distilled water ad libitum.

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# MICROSCOPICAL STUDY OF CARIES OF RATS' TEETH

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## INTRODUCTION

IN 1932 Hoppert, Webber, and Canniff[1] reported that they had been able to produce dental caries in the teeth of the albino rat, the cavities of which were visible to the naked eye. This was accomplished in 16 weeks on a diet containing ground corn. They stated that the retention of food particles of a particular size and consistency by the teeth seemed to be the determining factor in the production of cavities. Bibby and Sedwick[2] shortly afterwards stated that cavities in the teeth of rats fed on a synthetic diet consisting of ground yellow corn, wheat glutin, calcium carbonate, and sodium chloride originated as fractures of a portion of a cusp or fissure wall. Rosebury, Karshan, and Foley,[3] a few months later, reported that they had produced two types of carious lesions in the rat. These authors described "typical dental caries of molar fissures" and "cuspal dentin fractures followed by secondary caries-like changes." Other authors since then have made similar statements after observing, with the naked eye or under low magnification, the teeth of the albino rat fed on a ground corn or rice-containing diet. In 1944 Shaw, Schweigert, Elvehjem, and Phillips[4] described the histologic appearance of caries in the cotton rat molar tooth. They stated that the caries occurred almost exclusively deep in the occlusal fissures, and that it began at such a depth that early lesions were not evident on inspection of intact teeth. It was their opinion that when decay had progressed considerably, fracture of portions of the undermined cusp would occur. In 1945, Van Huysen and Vincent[5] stated they believed that a caries-producing diet of corn particles will not cause fracture unless the cusp is already weakened by caries. In a study by Shaw[6] in 1947 it was shown that, in comparison to the white rat,[8] the extraction of maxillary molars in the cotton rat had relatively little effect on the initiation and progress of carious lesions in the molar teeth subject to a corn particle diet. The reason for this difference has as yet not been explained. In 1949 Shaw[7] demonstrated in the cotton rat the fact that there was no evidence of mechanical injury of caries-susceptible enamel surfaces previous to the initiation of caries. In spite of this evidence with experimental caries and our experience in the clinic showing that microscopic lesions precede and are responsible for gross cavitation, one can still see occurring in the literature references to fracture type caries. Statements to the effect that rat caries is not true caries or that the teeth of the albino rat develop two types of caries, "fracture caries" and "fissure caries," are still being made.

Considering the teeth as a biological entity, their structure and function throughout the animal kingdom, it is difficult to believe any tooth could, unless weakened in some way, ordinarily become broken by chewing particles of corn.

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It is true that the enamel plates of the molar teeth of the rat are free at their occlusal ends, and the enamel plates distal to the fissures in the mandibular molars appear to have a mesial inclination, and those in the maxillary molars mesial to the fissures a distal inclination. The enamel plates and cusps are, however, functionally in line and parallel with the principal forces of masticatory stress. Some slight fracturing of the occlusal edges of the enamel does occur as attrition removes the exposed occlusal dentin supporting this marginal enamel. This is common to most herbivorous and omnivorous teeth. This fractured occlusal enamel is, however, relatively slight and involves only the enamel margin. The fractured areas still remain self-cleansing, and, therefore, do not decay.

The problem under discussion here is not this minute marginal enamel fracturing that is a result of attrition, but gross breaks of large portions of enamel plate or whole cusps with dentin and enamel involved so that retentive carious areas are formed. Nature usually provides more than adequate physical characteristics to protect stress-bearing tissue from ordinary stress. It hardly seems possible that such an ordinary food as corn, in spite of its relative hardness, would cause physical damage to enough teeth to account for the relatively high incidence of caries in certain teeth in the rat. This study, therefore, was set up to produce and observe under the microscope early tooth decay in the teeth of the albino rat. It was the purpose of these efforts to demonstrate and emphasize the fact that caries precedes, not follows, tooth fracture.

## METHOD

Fifty albino rats which were 25 days old were placed on a Hoppert, Webber, and Canniff diet.[1] Since the objective of this study was to examine the teeth before caries could become extensive enough to undermine or appreciably weaken the enamel or cusp dentin, the animals were kept on the diet for 31 days instead of the usual 100 days. At the end of 31 days the animals were killed, the heads were cut in half along their midsaggital plane, and fixed in 10 per cent neutral formalin. Four days later the heads were washed overnight in tap water. All 600 molar teeth of the 50 rats were examined for cavities. There was no evidence of caries or tooth fracture visible in any of the teeth, even when examined with the assistance of a binocular dissecting microscope at a magnification of 17 times. This was, of course, expected since the rats were on the cariogenic diet only 31 days after weaning. Following this examination for cavities, the half heads were dehydrated successively for 48 hours each in 50, 70, and 95 per cent alcohol solutions. When dehydrated, they were cleared in xylol and trimmed so that groups of 3 molar teeth were adequately supported by bone and soft tissue. These blocks of tissue-containing teeth were infiltrated with Canada balsam. The balsam-infiltrated jaws and molar teeth were mounted on a specimen holder and surface ground with a slurry of flour of pumice on a motor-driven machine. By changing the position of the tissue on the specimen holder once, it was possible to grind the teeth and jaws both on the lingual and buccal surfaces. The molar teeth of the albino rat are 2 mm. wide in their buccolingual dimension. The width of the teeth in the ground sections used

here was reduced by grinding first on the buccal and then on the lingual side, to less than 0.9 mm. (between 700 and 900 microns). An attempt was made to approach the centers of the first and second molars of each jaw. These sections were obviously very thick, but the grinding did make it possible to examine with brilliant transillumination the fissure contents at 90 times magnification. The ground sections of these cut molar teeth were examined for the following: fractured enamel, fissure contents, and caries.

Fig. 1.—Noncarious fissure showing plug of debris.

## OBSERVATIONS

It was pointed out above that no cavities were seen in the teeth examined with the naked eye and with the dissecting binocular microscope at 17 times magnification. This inspection was made upon all 600 of the available molar teeth of the 50 rats used in the experiment. After the ground sections were prepared and mounted, it was possible to examine them with brilliant transmitted light. In Fig. 1, a well-defined fissure with clearly delineated dentin and enamel junction may be seen. In most instances there was, as is shown in all of the illustrations, perceptible buccolingual depth of the fissures. Two hundred eighty-five of these rat molar fissures were examined. These teeth were not, of course, all cut at the same buccolingual plane, but in each recorded instance, a fissure was clearly visible. In 85 per cent of the 285 fissures examined, there was visible microscopically a plug of material that was reddish-tan in color and somewhat fibrous in texture.[6] In spite of the diet and rigorous procedures necessary to grinding the sections, in only one instance, which is not shown here, was there any appreciable loss of cusp enamel. The exposed tissue of this fracture was not carious, so it was assumed that the change had been brought about during the grinding of the section.

In 165 of the 285 fissures examined under the microscope, there was visible in the enamel at the pulpal end or curvature of the fissure a small crack shown in Fig. 2. These small vertical cracks have been previously called lamellae. They have been described as areas of hyperkeratinization and designated as pathways for the penetration of the carious-surface lesions into the dentino-enamel junction.[3] In the sections examined in this study these cracks also appear in other parts of the enamel. In no instance, however, were any of these breaks associated with caries. It is this writer's opinion that they were artifacts brought about by the drying and shrinking of the dentin supporting the enamel.

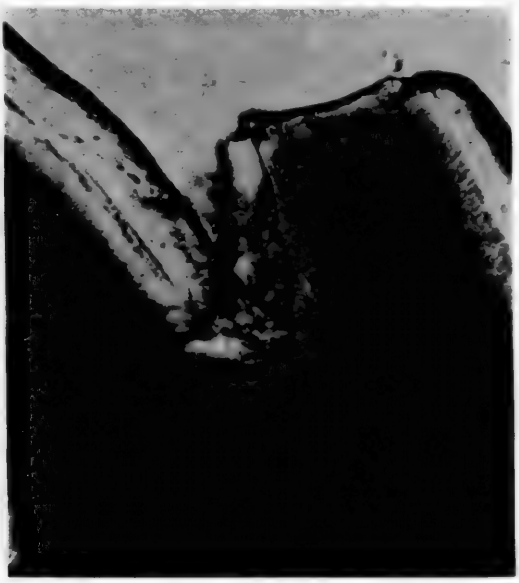

Fig. 2.—Fissure without impacted food. There is a fine crack in the enamel at the pulpal end of the fissure.

One can see in Fig. 3 that the caries has attacked the enamel which is still in position. The enamel caries occurred just occlusal to the apex or pulpal end of the fissure, not at the closed end.[6] The carious enamel was dark brown and had lost its translucency, becoming very opaque. This brown pigmentation extended into the dentin. It was apparently directed towards the nearby pulp. It is also evident in the illustration that further penetration of the caries along such a pathway would undermine the cusp and result in its fracture. This picture is the one usually seen in caries of rat fissures and accounts for the so-called fracture caries described grossly at 100 or more days. The horizontal plug in the more occlusal portion of the fissure in Fig. 3 appeared under the microscope to be a piece of metal impacted in the fissure. The darkened areas in Fig. 4 show another instance of early caries of the enamel and dentin. Both fissures contain food plugs, while only one side shows evidence of caries. Rosebury, Karshan, and Foley[3] and Sognnaes[9] have given excellent descriptions of caries at this stage in the albino rat.

COMMENT

Because of the physical nature of the corn particles in the diet, the anatomical structure of the tooth and the fact that the incidence of caries in the rat has been studied mostly under low magnification or with the naked eye, it has been inaccurately reasoned that the initiation of caries in the rat is due to fracturing of the tooth and therefore different than that in man. Most any

Fig. 3.—Caries of the enamel walls near but not at the pulpal end of the fissure. It is directed toward the pulp horns.

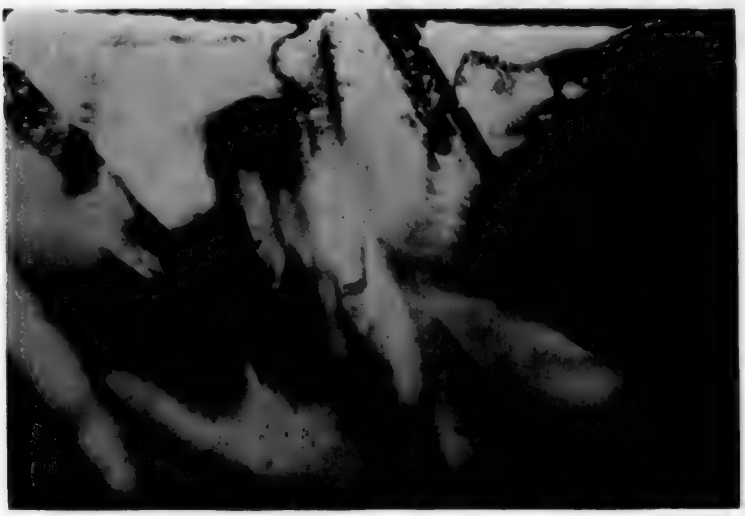

Fig. 4.—Both fissures show plugs. The mesial fissure shows caries. There is spreading of the dentin caries toward the pulp.

textbook describing dental caries will point out the undermining effects of caries in man and the fact that grossly visible cavities are brought about by fracturing. This fracturing occurs late in the carious process whether it be in man, the albino rat, or the cotton rat. It is paradoxical that the dental practitioner should advise patients to see their dentists often so that cavities may be discovered early and not after extensive and dangerous undermining and fracture; yet the dental scientist will derive profound conclusive statements, not on dental caries but upon artifacts brought about by the late stages of the carious process. Although this study still does not describe how caries is initiated in the rat molar, it does show that caries in the rat's molar, like any other tooth, precedes cavitation and that any fracturing of the tooth is due to the undermining destruction of the tissue characteristic of dentin caries anywhere.

## CONCLUSION

These findings add more weight to the importance of rat caries in the study of this disease. There is no question but that for the further elucidation of the question of the role of physical vs. chemical, dietary factors will have to be made using earlier microscopical rather than late gross methods of examination. The fracturing is secondary to the caries. Lamellae are artifacts produced during processing of tissue for slides and do not contribute to the destruction of the tooth by caries.

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# THE BASIC AMINO ACID CONTENT OF ENAMEL PROTEIN

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LOSEE and Hess[1] have reported the cystine, methionine, and phenylalanine content of human enamel protein. These amino acids, however, are not indicative of the nature of the protein. It is commonly believed that enamel protein is a keratin and the basis for this belief has been adequately reviewed by Block, Horwitt, and Bolling.[2] These investigators used the criteria, previously established by Block and Bolling,[3] that eukeratin contains the basic amino acids, histidine, lysine, and arginine, in the approximate mol ratios of 1:4:12. Using paper chromatography Block, Horwitt and Bolling[2] found that enamel protein contains 0.9 per cent histidine, 2.8 per cent lysine, and 9.7 per cent arginine, calculated to 16.0 per cent nitrogen in the enamel protein, these values give mol ratios of 1:3:10. We have determined the same three amino acids by a variety of methods and the results are the subject of this paper.

Three different methods of analysis have been employed for the determination of some or all of the three basic amino acids: paper chromatography, chromatographic separation using a mixture of Lloyd's reagent and Super-Cel, and microbiologic assay. The determination by paper chromatography, using water-saturated phenol, was by the procedure of Consden, Gordon, and Martin[4] except that the strips were run against gravity with the phenol at the bottom of the vessel. The amount of histidine was measured by color development with the diazo reagent of Urbach[5] and comparison with strips containing known amounts of histidine. The lysine values were determined, after color development with a 0.5 per cent solution of ninhydrin in butyl alcohol saturated with water, by comparison with strips containing known amounts of lysine.

The chromatographic separation of the basic amino acids devised by Bergdoll and Doty[6] was employed with a slight modification. We found that their mixture of Lloyd's reagent and Hyflo Super-Cel gave a column that permitted exceedingly slow elution of the several fractions. However by mixing equal parts of washed sand with the mixture, the elution can be speeded up considerably. In the fractions thus obtained histidine was determined by the method of Macpherson,[7] the arginine by the method of Thomas, Ingalls, and Luck,[8] and the lysine by the procedure of Bergdoll and Doty.[6] The microbiologic assays were run on hydrolysates of enamel protein prepared as described by Losee and Hess.[1] These assays were carried out at the Microbiological Laboratory at the National Institutes of Health.

The enamel protein, prepared from noncarious fully erupted permanent first and second molars by the dialysis procedure previously described,[1] contained

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13.59 per cent nitrogen. The values given in Table I for histidine, arginine, and lysine are calculated on the weight of the enamel protein dried in a desiccator over calcium chloride for 48 hours.

TABLE I

HISTIDINE, LYSINE, AND ARGININE CONTENT OF ENAMEL PROTEIN

| AMINO ACID | METHOD OF DETERMINATION | | |
|---|---|---|---|
| | PAPER CHROMAT. (%) | BERGDOLL AND DOTY CHROMAT. (%) | MICROBIOLOGIC (%) |
| Histidine | 1.0 | 0.70 | 1.15 |
| Lysine | 2.7 | 2.22 | 3.75 |
| Arginine | | 6.92 | 6.72 |

The results obtained by paper chromatography are probably the least accurate, the results by the other two methods are of a higher order of accuracy. The microbiologic value for lysine is higher than that obtained by the chromatographic separation; however, it can be noted that the paper chromatogram value is close to the latter figure. The values determined by the method of Bergdoll and Doty[6] calculated to 16.0 per cent nitrogen are 0.88, 2.82, and 8.78 per cent for histidine, lysine, and arginine, respectively. These figures are in excellent agreement with those reported by Block, Horwitt and Bolling[2] on a sample of enamel protein that contained 11.9 per cent nitrogen.

The mol ratios for histidine, lysine, and arginine calculated from the amounts obtained by the chromatographic separation are 1:3:9, in close agreement with 1:3:10 calculated from the values of Block, Horwitt and Bolling,[2] and approximate the ratio of 1:4:12 set by Block and Bolling[3] as the criteria for an eukeratin.

## SUMMARY

The histidine, lysine, and arginine content of enamel protein prepared from noncarious fully erupted permanent first and second molars has been determined by paper chromatography, chromatographic separation upon a mixture of Lloyd's reagent and Super-Cel, and by microbiologic assay. The mol ratios of these three amino acids are 1:3:9, in fairly close agreement with the ratios of 1:4:12 suggested for an eukeratin.

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# CHANGES IN ALKALINE PHOSPHATASE ACTIVITY OF RAT ORAL EPITHELIUM DURING THE ESTROUS CYCLE AND IN RESPONSE TO ADMINISTERED ESTROGEN

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IT HAS been reported that estrogen deficiency states in women may lead to regressive alteration of the oral mucosa.[1, 2] This observation is supported also by animal experimentation.[3] The degenerative changes can be reversed by local or systemic administration of estrogenic hormone, the response involving hyperplasia and hyperkeratinization of the oral epithelium.[1, 4, 5]

Estrogens have been shown to influence alkaline phosphatase activity in the vagina.[6, 7] It has been postulated that in the vagina the enzyme is concerned with the chemistry of keratin formation.[7]

The present paper utilizes the alkaline phosphatase visualization method as a measure of the activity of oral epithelium in response to stimulation by fluctuating, physiologic quantities of estrogen during the normal estrous cycle of the rat. The physiologic extremes of estrogen deficiency and supply are further magnified in ovariectomized animals and in spayed animals administered large amounts of exogenous hormone.

## METHOD

A large group of normal albino female rats, subsisting on a routine dog chow diet, was examined by daily vaginal smears during 3 or 4 estrous cycles. Eighteen animals which had exhibited normal cycles were sacrificed: 5 each in diestrus, proestrus, and estrus and 3 in metestrus.

Five female rats spayed for periods of from 2 to 5 weeks were injected with estradiol benzoate in oil. The injections were made over a 3-day period, the total amounts of injected hormone ranging from 0.15 to 0.5 mg. Five uninjected spayed females served as controls. The spayed animals were killed on the day following the last injection.

All animals were killed by decapitation. A strip of buccal mucosa, extending dorsally to the border of the hard palate between the incisor and molar teeth, was removed and fixed in cold 80 per cent ethyl alcohol. The interdental papilla between the incisor teeth was also excised and similarly treated.

The tissues were sectioned in paraffin at 7 microns and alternate sections stained by the alkaline phosphatase technic of Gomori[8] (modified by the addition of 0.1M $MgSO_4$ to the incubating medium) or with Harris hematoxylin and eosin. The alkaline phosphatase preparations were incubated in glycerophosphate at pH 9.4 and 37° C. for periods of ½, 1, 2, and 24 hours. Control sections were similarly incubated in a solution lacking glycerophosphate.

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## RESULTS

A careful study of routine (hematoxylin-eosin) preparations of buccal mucosa fails to reveal any constant correlation between estrogen level and oral epithelial histology. At all stages the buccal mucosa is keratinized, though not to the same extent in all parts of a given section. Thus, neither the thickness of the epithelium, thickness of the stratum corneum, or any other observed histologic or cytologic detail permits the differentiation of oral mucosae from spayed, estrous, or estrogen-treated rats under the conditions operating in this study.

Alkaline phosphatase preparations of buccal mucosa on the other hand show a graded response to blood level of estrogenic hormone cyclically released by the ovary or administered parenterally. The observed differences are quantitative, not qualitative, in nature.

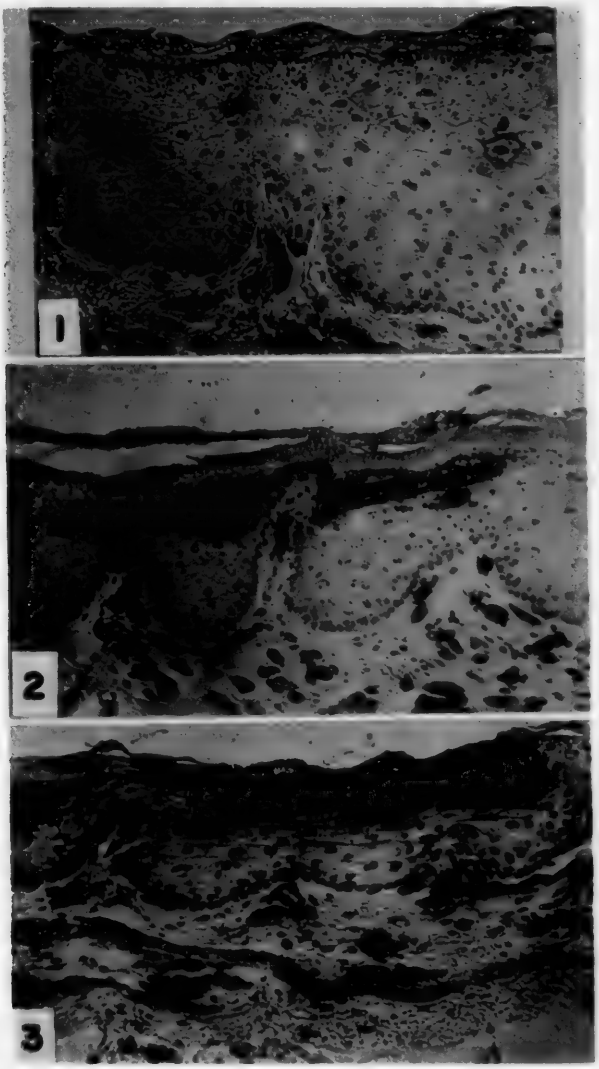
In none of the diestrous rats nor in uninjected ovariectomized animals is alkaline phosphatase activity completely eliminated from the epithelium. With short periods of incubation (½ to 1 hour), precipitated cobalt sulfide (representing sites of activity of alkaline phosphatase) is very lightly but, in general, uniformly distributed through the epithelium. In contrast the superficial blood vessels are intensely blackened. There is a slightly darker staining of the nuclei of basal cells, of large keratohyaline granules, and of the cornified band. Specimens from 2 of the diestrous animals (but none of the castrates) exhibit scattered zones of more pronounced darkening with the precipitate tending to outline individual cells. These sites of apparently greater phosphatase activity correspond to areas in routine preparations in which there is an abundance of keratohyaline granules.

With prolonged incubation the general staining (cobalt sulfide precipitation) pattern is similar but intensified. Thus at 24 hours' incubation (Fig. 1) there is a faint background coloration of the cytoplasm, accentuated in the stratum corneum, a distinct staining of the basal cell nuclei, a less intense precipitation in the nuclei of cells in the intermediate layers, and a marked blackening of the larger keratohyaline granules. With 24 hours' incubation, also, more areas are observed of apparently augmented phosphatase activity as evidenced by the greater number of cells, isolated or in small groups, outlined by cobalt sulfide precipitation at the periphery of the cells and in the intercellular spaces (Fig. 2). Such areas are observed in buccal epithelium of most of the diestrous and spayed animals with longer periods of incubation. It should be emphasized that activity of the enzyme is measured both by the intensity of the visible reaction and by the time required for the reaction to develop.

In proestrus (Fig. 3) and estrus (Figs. 5, 7, and 9) the number and extent of stained areas indicative of high enzymatic activity are increased. In the tissues from normal estrous animals the described zones of activity are observed with only one-half hour of incubation.

The relation of alkaline phosphatase to keratin formation is suggested by the distribution of the bands of pronounced cobalt sulfide deposition. In

areas of mature keratin (Fig. 4) strong phosphatase activity is limited to a few cells and keratohyaline granules immediately underlying the corneum (Fig. 5). In regions which are suggestive of more active keratinization (Fig. 6), precipitation indicating marked phosphatase activity is more extensive, being observed even in the cornified layer itself (Fig. 7). Some areas of the cheek (Fig. 8) show evidence of continuous keratinization, possibly as the result of




Figs. 1-13.—The illustrations are unretouched photomicrographs taken at an original magnification of about ×175. Alkaline phosphatase preparations incubated 24 hours.

Fig. 1.—Buccal mucosa from normal diestrous rat. Phosphatase preparation.

Fig. 2.—Buccal mucosa from uninjected spayed rat. Areas of high enzymatic activity alternate with zones relatively devoid of alkaline phosphatase. Phosphatase preparation.

Fig. 3.—Buccal mucosa from normal proestrous rat. Phosphatase preparation.

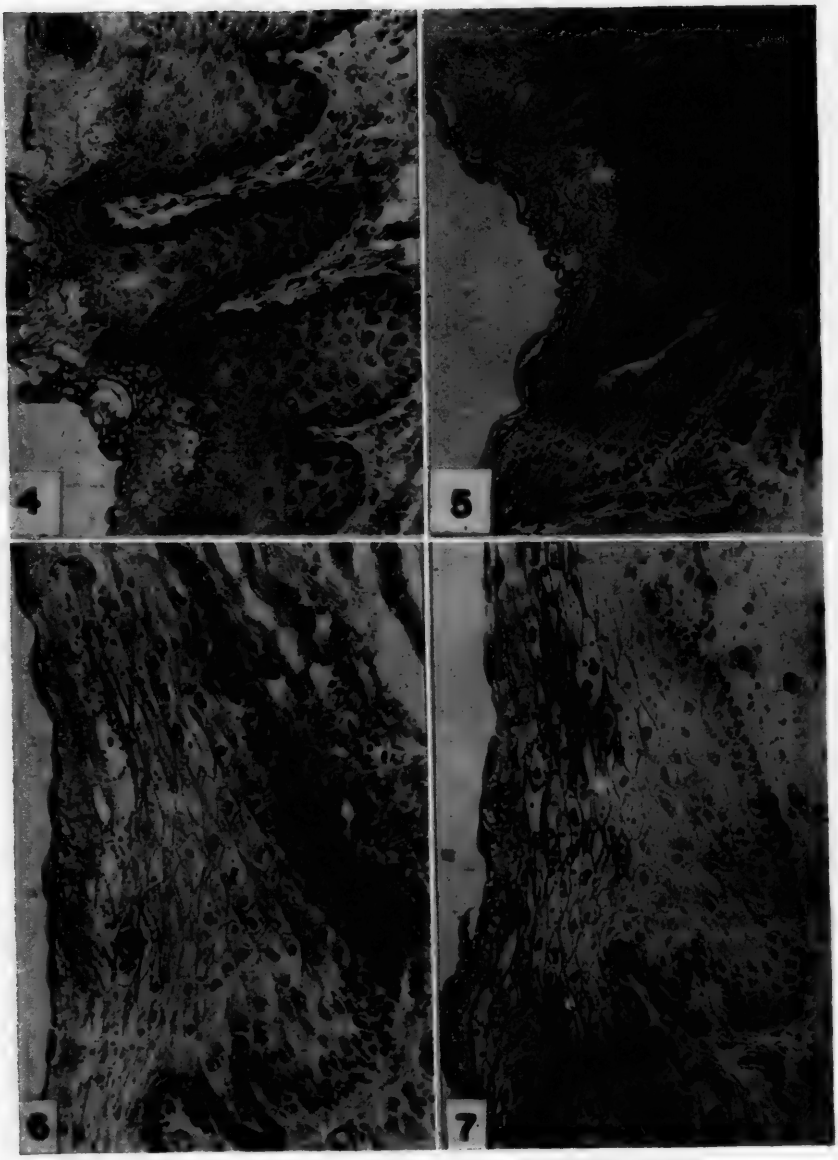



Fig. 4.—Buccal mucosa from normal estrous rat. Area of mature keratin. Hematoxylin and eosin.

Fig. 5.—Same animal and region as in Fig. 4. Phosphatase preparation.

Fig. 6.—Buccal mucosa from same animal as in Fig. 4, but adjacent area showing actively keratinizing epithelium. Hematoxylin and eosin.

Fig. 7.—Same animal and region as in Fig. 6. Phosphatase preparation.

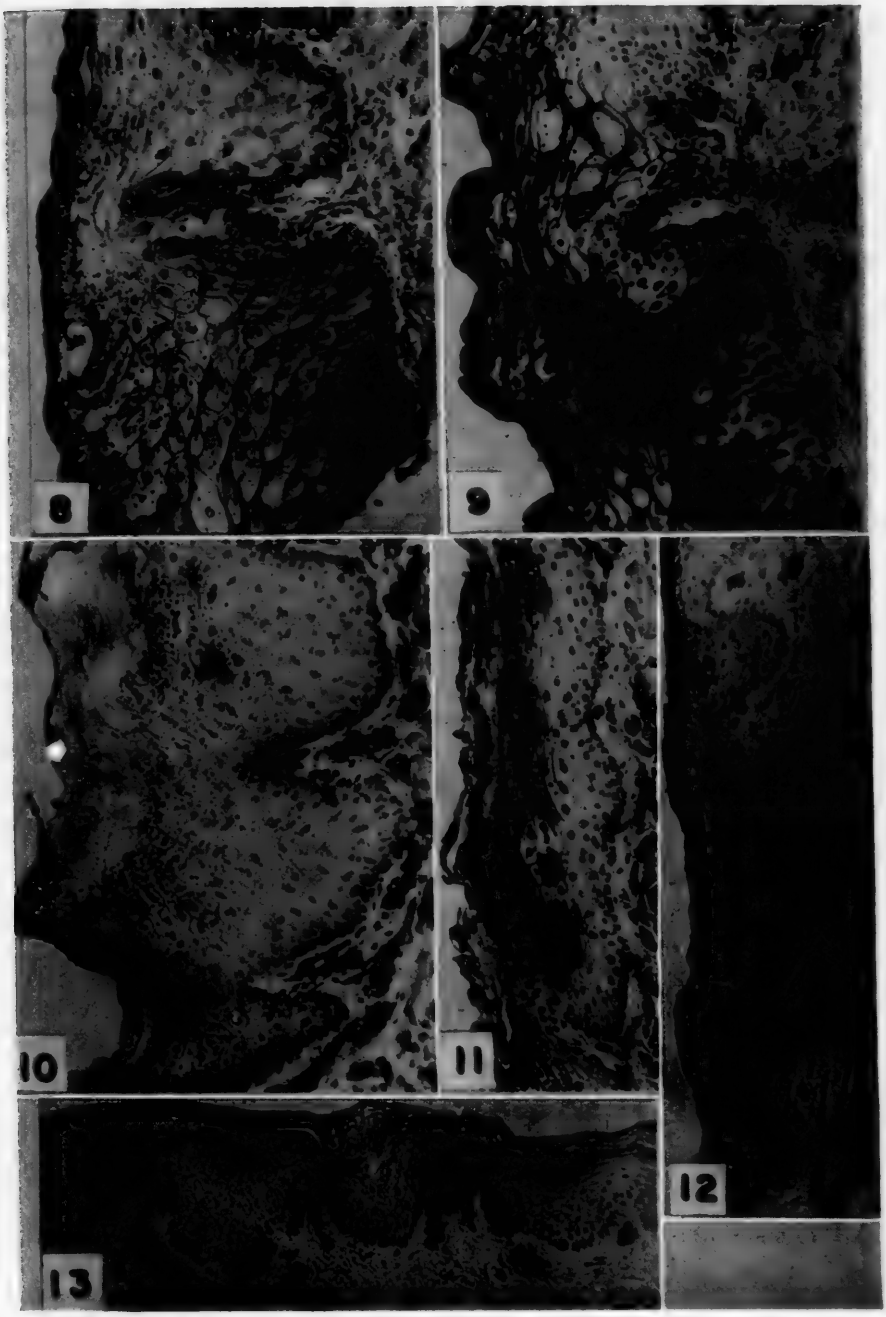



Fig. 8.—Buccal mucosa from same animal as in Figs. 4 to 7 but adjacent area showing more pronounced active cornification. Hematoxylin and eosin.

Fig. 9.—Same animal and region as in Fig. 8. Phosphatase preparation.

Fig. 10.—Buccal mucosa from spayed rat injected with 0.15 mg. estradiol benzoate. Phosphatase preparation.

Fig. 11.—Buccal mucosa from spayed rat injected with 0.5 mg. estradiol benzoate. Phosphatase preparation.

Fig. 12.—Buccal mucosa from normal metestrous rat. Phosphatase preparation.

Fig. 13.—Gingival mucosa from interdental papilla between incisor teeth of rat in normal estrus. Animals in other stages of cycle and estrogen-injected animals exhibit similar picture. Dark band at surface is oral detritus. Phosphatase preparation.

physical influences operating locally. Alkaline phosphatase activity here appears to be high throughout the epithelium as deep as the basal cell layer (Fig. 9).

Administration of estrogen to spayed rats appears to increase alkaline phosphatase activity of buccal mucosa in all animals studied. It is again noted that heavy cobalt sulfide deposition is discontinuous, and zones of heavy precipitation are observable after only one-half hour's incubation. In general, the epithelium underlying mature keratin (Fig. 10) exhibits less precipitate than areas which seem to be undergoing active cornification (Fig. 11). Higher doses of exogenous estrogen (0.5 mg. estradiol benzoate) are correlated with a more pronounced keratin layer but less alkaline phosphatase activity, whereas smaller quantities of estrogen (0.15 mg.) result, in general, in a thinner corneum but more demonstrable activity of the enzyme.

Buccal epithelium of metestrous rats exhibits a distribution of cobalt sulfide indicative of a shifting of alkaline phosphatase activity toward the surface (Fig. 12). At this stage, which is related to a rapidly falling blood estrogen level, large areas of the epithelium are devoid of enzymatic activity other than that constantly present in this tissue and thus resemble closely the diestrous state.

Gingival epithelium, as seen in sections of interdental papilla, exhibits a uniform picture regardless of estrogen level. Alkaline phosphatase activity is negligible with short periods of incubation. At 24 hours cobalt sulfide is deposited in nuclei of the deeper cells, faintly in the cytoplasm of all cells including the superficial cornified plates, and more heavily in the cells of the well-defined stratum granulosum (Fig. 13). Superficial blood vessels show the characteristically high acitvity of the enzyme.

## DISCUSSION

On the basis of the distribution of active phosphatase enzyme in the cornifying vaginal membrane of the rat, Ring[7] postulated a functional relationship between the local activity of this enzyme and keratin formation. Kamell and Atkinson[6] have reported a similar distribution of alkaline phosphatase in the vaginas of spayed, estrogen-treated mice, with a parallel gradient in the concentration of cytoplasmic ribonucleic acid. Alkaline phosphatase and pentose nucleic acid have been implicated in the biosynthesis of proteins (see Moog[9]).

The keratinization of stratified squamous epithelium of mucous membranes can be related to a number of influences: nutritional, mechanical, and hormonal. It is our opinion that the first two factors operated uniformly in all groups in this study. Observed differences between groups of animals can thus be regarded as resulting from changes in the hormonal state, specifically changes in blood level of estrogen.

Several investigators have shown that estrogen deficiency leads to regressive changes in oral epithelium.[1, 2, 3] Hyperplasia and hyperkeratinization of oral

mucosa have followed administration of estrogens.[1, 4, 5, 10, 11] These observations have pertained to women and female monkeys and dogs. The female rat has not hitherto been studied in this connection.

In the rat, as the result apparently of mechanical dietary factors, the oral epithelia are normally keratinized. Consistent changes in degree of keratinization could not be seen in routine preparations, although it should be noted that the greatest development of the cornified layer occurred in those spayed animals which received maximal dosages of estradiol benzoate. Similarly, no consistent hyperplastic growth of the oral mucous membrane was observed in the estrus or estrogen-treated groups. There was, in fact, a notable paucity of mitotic figures at all stages of the estrous cycle and in the estrogenized animal. Hypertrophy and binucleation of cells in the intermediate (prickle cell) layers were observed frequently without correlation with estrogen level.

It must be emphasized that increased alkaline phosphatase activity of buccal epithelium with increasing estrogen level was not a uniform condition as demonstrated histochemically. At all stages, areas of epithelium were present presumably devoid of any but what might be termed "background" activity of this enzyme (faint staining of nuclei and cytoplasm somewhat intensified in the basal cell layer, in the cornified layer, and in keratohyaline granules). Zones of marked activity were superimposed, discontinuously. These zones of augmented enzymatic activity increased in number, and the time necessary to develop the visual reaction diminished, with increase in estrogen. It is well known that circulating estrogen is low in metestrus and diestrus and increases to a maximum during proestrus and estrus.

The discontinuity in distribution of alkaline phosphatase activity suggests an intermittent process of keratin formation. This peculiarity of distribution was also observed in vaginal epithelium[7] but was less marked in that tissue. The distribution of zones of high enzymatic activity in relation to maturity of keratin tends to confirm the postulated causal relationship between alkaline phosphatase and cornification. A maximally keratinized membrane (or portion of membrane) will under normal circumstances not cornify further until the corneum is reduced by attrition. Thus, phosphatase activity was, in general, low in areas underlying thick bands of mature keratin but high in areas suggestive of active keratinization. Similarly large quantities of injected estrogen resulted in more mature keratin and less demonstrable phosphatase activity, whereas smaller quantities had a converse effect. Presumably the larger dose produced a rapid and complete keratinization prior to the time the tissues were removed.

Gingival epithelium was found to be relatively devoid of alkaline phosphatase, confirming the prior observation of Zander.[12] In this connection the gingivae are similar to skin, which also exhibits a negligible activity of this enzyme. Biesele and Biesele[13] have described an augmented phosphatase reaction in epidermis of mice painted with methylcholanthrene. Fell and Danielli[14] found the activity of alkaline phosphatase increased in healing cutaneous wounds experimentally produced in rats. It is possible that gingival

and cutaneous epithelia, both of which are normally fully cornified, maintain their keratinized state with levels of phosphatase below that necessary for histochemical demonstration.

SUMMARY

1. The alkaline phosphatase visualization method of Gomori affords a means of measuring at least one aspect of the chemical activity of cells in the oral mucous membranes in the absence of marked morphologic change.

2. Alkaline phosphatase activity of buccal epithelium increases during the estrous cycle of the normal female rat from a minimum in diestrus to a maximum during estrus.

3. The extremes are amplified in the spayed and spayed-estrogen treated animals.

4. Gingival epithelium exhibits a negligible phosphatase activity during all stages of the estrous cycle and in the presence of administered estrogen.

5. The distribution of zones of activity of this enzyme in buccal epithelium suggests a relationship to the process of keratin formation.

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# THE USE OF CHLORAMPHENICOL (CHLOROMYCETIN) FOR KILLING SOME PENICILLIN-STREPTOMYCIN RESISTANT ORGANISMS ENCOUNTERED IN INFECTED ROOT CANALS

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PREVIOUS investigations[1, 2] have demonstrated that the combination of penicillin and streptomycin is capable of sterilizing all infected root canals with the exception of those infected with either fungi or streptococci of the enterococci group. Our previous in vitro tests[3] showed that neither penicillin nor streptomycin was able to kill the fungi which are present in about 19 per cent of all infected root canals. The enterococci, which are present in about 6 to 8 per cent of the cases, have also been shown to be resistant to penicillin and streptomycin in vivo.[4]

It is obvious, therefore, that if we are to obtain a method of treatment which is successful in all cases, other antibacterial agents must be added to the mixture. Our research program has, therefore, been directed toward three main objectives: (1) In vitro tests of various antibiotics to find those which are effective against the enterococci. (2) In vitro tests of fungicides which are effective against the Candida group. (3) Clinical application of those agents which appear to be effective in vitro, so that their effectiveness may be appraised.

The procedures described below attempt to accomplish our first two objectives, namely, tests of various antibiotics against the enterococci and fungi.

## EXPERIMENTAL

### *In Vitro Tests of Antibiotics Against Enterococci*

Three of the 4 known groups of enterococci were isolated from infected root canals. One strain each of *Streptococcus zymogenes, Streptococcus faecalis,* and *Streptococcus liquefaciens* was isolated from infected root canals and grown on brain-heart infusion broth (Difco). Each culture was then treated in the following manner: To 4.5 ml. of brain-heart infusion broth, 0.5 ml. of a 24-hour culture was added. This made a 1 to 10 dilution of organisms ($10^{-1}$). To this tube a known quantity of antibiotic was added. The tube was then incubated for 24 hours. At the end of this time, a subculture of 0.1 ml. from the tube into 100 ml. of brain-heart infusion broth was made. The subcultures were allowed to incubate for 1 week before results were noted. Antibiotics tested were: (1) potassium penicillin G., in strengths ranging from 50,000 units per ml. to 500 units per ml.; (2) streptomycin, in strengths ranging from 25 mg. per ml. to 0.0025 mg. per ml.; (3) combinations of these 2 antibiotics at various strengths (Table I), and (4) chloramphenicol (Chloromycetin) in strengths ranging from 25 mg. per ml. to 0.32 mg. per ml.

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This method of testing has previously been described.[3] It is a critical test, since if one bacterium survives, a positive subculture will result. It is also comparable to the clinical method of taking a culture from a root canal. In endodontia we are primarily interested in bactericidal not bacteriostatic action.

*Results.*—As can be seen from Table I, penicillin was not effective until the high concentration of $>$ 5,000 and $<$ 25,000 units per ml. was used against each organism. Likewise, the bactericidal action of streptomycin against all

TABLE I

EFFECT OF ANTIBIOTICS ON THE ENTEROCOCCI

| | STR. FAECALIS | STR. ZYMOGENES | STR. LIQUEFACIENS |
|---|---|---|---|
| Penicillin (units per ml.) | | | |
| 50,000 | 0 | 0 | 0 |
| 25,000 | 0 | 0 | 0 |
| 5,000 – 25.0 | + | + | + |
| 2.5 | + | + | + |
| Streptomycin (mg./ml.) | | | |
| 500 | 0 | 0 | 0 |
| 250 | 0 | 0 | 0 |
| 50 | + | + | + |
| 25 | 0 | 0 | + |
| 5.0 | + | + | + |
| 2.5 | + | 0 | + |
| 0.25 | + | + | + |
| 0.025 | + | + | + |
| Penicillin (units per ml.) and Streptomycin (mg. per ml.) | | | |
| P S | | | |
| 25,000 250 | 0 | 0 | 0 |
| 5,000 50 | + | 0 | + |
| 500 50 | + | 0 | + |
| 50 0.5 | + | + | + |
| 5 0.05 | + | + | + |
| Chloramphenicol (mg./ml.) | | | |
| 25.0 – 0.32 | 0 | 0 | 0 |

0 = no growth.
+ = growth.

3 organisms was not decisive until a level of $>$ 50 and $<$ 250 mg. per ml. was used. Combinations of penicillin and streptomycin did not exhibit a synergistic effect against the test organisms. Bactericidal action was not evident against 2 of the 3 organisms, *Str. faecalis* and *Str. liquefaciens,* until $>$ 5,000 and $<$ 25,000 units of penicillin and $>$ 50 and $<$ 250 mg. of streptomycin were employed.

Chloramphenicol (Chloromycetin), on the other hand, exhibited a bactericidal action against all 3 organisms at all strengths tested (250.0 to 0.32 mg. per ml.). Bacteriostatic tests showed sensitivity of the enterococci to chloramphenicol at 12.5 $\mu$g./ml.

Aureomycin, which exhibited a bacteriostatic effect against the enterococci at 0.098 $\mu$g./ml., was not tested because of its instability. When dissolved, it

begins deteriorating within a few hours. It would, therefore, not be a desirable agent for use in root canal therapy unless it were used freshly dissolved for each treatment. Economically, it would be better to have a stock mixture of antibiotics which is stable at room temperature.

*In Vitro Tests of Antibiotics by Paper Disc Agar Diffusion Method*

A pure strain of *Str. faecalis* was isolated from an infected root canal and grown on brain-heart infusion broth. One-tenth milliliter of this culture was inoculated into 10 ml. of melted blood agar and a plate was poured. This was repeated 6 times. Discs of Whatman filter paper were then moistened with the various antibiotics to be tested. These discs were placed one on each plate and the plate was incubated. At the end of 24 hours, the zone of inhibition was measured. The same procedure was repeated using cultures of *Str. zymogenes* and *Str. liquefaciens*. The following agents were tested: streptomycin 1 Gm. per 2 ml. propylene glycol; penicillin, 1,000,000 units per 2 ml. propylene glycol; Chloromycetin, 1 Gm./2 ml. propylene glycol; combination of penicillin 1,00,000 units and streptomycin 1 Gm. per 2 ml. propylene glycol; combination of penicillin, 1,000,000 units, streptomycin 1 Gm., and Chloromycetin 1 Gm., per 2 ml. propylene glycol; and propylene glycol.

*Results.*—Table II shows the sizes of the zones produced.

TABLE II

ZONES OF INHIBITION IN MILLIMETERS

| | PENICILLIN | STREPTOMYCIN | PENICILLIN STREPTOMYCIN | CHLOROMYCETIN | PENICILLIN STREPTOMYCIN CHLOROMYCETIN | PROPYLENE GLYCOL |
|---|---|---|---|---|---|---|
| *Str. Zymogenes* | 23 | 14 | 18 | 23 | 22 | 7 |
| *Str. faecalis* | 24 | 14 | 24 | 22 | 24 | 7 |
| *Str. liquefaciens* | 21 | 14 | 14 | 24 | 25 | 7 |

Streptomycin does not appear to be as effective as the other agents either singly or in combination. Both penicillin and Chloromycetin are effective and so are combinations involving these two antibiotics. The zones produced by the combination of penicillin, streptomycin, and Chloromycetin are no larger than those produced by Chloromycetin alone.

The paper disc agar diffusion method measures only the degree of bacteriostasis. Zones of inhibition give an indefinite measurement of antibacterial potency and should be used for screening purposes only. Examination of the above results illustrates this quite clearly. The zones of inhibition of penicillin and Chloromycetin are practically the same. However, clinically, these same strains were resistant to penicillin but susceptible to Chloromycetin.

*In Vitro Tests of Fungicides Against Candida*

This procedure was employed to test bacillomycin A,* a newer antifungal antibiotic, to see whether or not it was effective against an organism of the Candida group isolated from an infected root canal: To 0.5 ml. of a pure culture of the fungus was added 4.5 ml. of Sabouraud's broth. Serial dilutions

*Supplied by Wyeth Inc., Philadelphia, Pa.

were then made with Sabouraud's broth from $10^{-1}$ to $10^{-7}$. To each of the 7 tubes, a known quantity of bacillomycin A was added. The tubes were then incubated for 48 hours. After this, 0.1 ml. of each tube was subcultured in flasks containing 100 ml. of Sabouraud's broth. After incubation for 7 days, results were recorded.

*Results.*—Quantities of bacillomycin A, ranging from 250 mg./ml. to 25 mg./ml. were all ineffective in killing the test organisms even though the zone of inhibition was 30 mm.

## DISCUSSION

The tests revealed that penicillin and streptomycin both are effective against the enterococci if the concentrations of each are extremely high. At 25,000 units per ml. or over, penicillin will kill these organisms, under the in vitro conditions. Likewise, streptomycin is bactericidal above 250 mg./ml. Clinically, however, neither of these antibiotics was effective. This was demonstrated by the fact that the test organisms were each subjected to treatments from a mixture containing 1,000,000 units of penicillin and 1 Gm. of streptomycin per 2 ml. of propylene glycol. It is practically a physical impossibility to introduce 25,000 units of penicillin and/or 250 mg. of streptomycin into a root canal. The volume of an average upper first incisor root canal, for example, may range from 0.01 to 0.02 ml. Even assuming that the canal were filled completely with antibiotic, the amount introduced would still be far less than the effective killing dose. Less resistant strains of enterococci might possibly be killed with smaller doses of penicillin or streptomycin. However, penicillin deteriorates upon standing in propylene glycol at room temperature. Tests made by the Food and Drug Administration[5] revealed that at room temperature penicillin in propylene glycol loses 40 per cent of its potency in 7 days. The loss of potency of penicillin dissolved in propylene glycol paralleled the loss obtained when penicillin was dissolved in distilled water.

Streptomycin, on the other hand, is quite stable in propylene glycol at room temperature. Against less resistant strains of enterococci its clinical ineffectivesness is attributable to the fact that microorganisms develop resistance to streptomycin more easily than to penicillin. Unless a total killing dose is delivered into the root canal at one treatment, the original resistance of the organism is increased so that the amounts of streptomycin subsequently added are insufficient to kill.

Robbins and Tompsett[6] demonstrated that penicillin was therapeutically effective in subacute bacterial endocarditis in all but 10 per cent of the cases treated. The majority of the group of patients in which the infection was not arrested were infected with either *Str. faecalis* or *Str. zymogenes*. They also found the enterococci to exhibit considerable resistance to streptomycin. Penicillin and streptomycin, even when acting together, exhibited no synergistic effect against the enterococci.

As a result, it is necessary to add another antibiotic to the combination for effective action against all bacteria. Among the available antibiotics which are

effective against the enterococci are chloramphenicol, aureomycin, bacitracin, tyrothricin, and the polymixons. The last four named exhibit certain undesirable properties, such as instability and toxicity, which limit or contraindicate their utilization in endodontic treatment.

Chloramphenicol, on the other hand, appears to be almost an ideal antibiotic and extremely well suited for use in root canal therapy. Dissolved in propylene glycol, it is stable for an indefinite period at room temperature. As our tests indicated, it is effective against certain microorganisms which are resistant to penicillin and streptomycin. In addition it has specific therapeutic activity against a wide variety of pathogenic organisms. It is soluble in propylene glycol and also compatible with other antibiotics.

As a result of these tests, the addition of chloramphenicol to a mixture of antibiotics is both indicated and desirable.

The only group of organisms now left uninhibited by our combination of antibiotics are the fungi. We are seeking an antibiotic with antifungal properties which can be incorporated in our mixture to complete the antimicrobial spectrum of the infected pulpless tooth. Tests with bacillomycin A, a newer antifungal antibiotic,[7] showed that it was fungistatic, but not fungicidal, for *C. albicans* at 250 mg./ml. or less. There appears to be quite a similarity between bacillomycin A and antibiotic XG.[8] Both possess polypeptide characteristics. Antibiotic XG was tested clinically in 5 cases infected with a fungus. In the strength supplied by the manufacturer (2000 units/ml. propylene glycol) it was not effective.

It is apparent, therefore, that at the present time, an antifungal antibiotic for use in endodontic procedures is not available. Until one is developed, a chemical fungicide will have to be added to the mixture. Our investigation at the present time is directed toward that end. Once an effective fungicide, compatable with the antibiotics is found, a negative culture after one treatment will be realized.

## SUMMARY AND CONCLUSIONS

1. Penicillin is bactericidal for the enterococci at $> 5{,}000$ and $< 25{,}000$ units per ml. propylene glycol, in vitro.

2. Streptomycin is bactericidal for the enterococci at $> 50$ and $< 250$ mg./ml. in vitro.

3. In vitro tests show that chloramphenicol is highly effective against the enterococci at strengths ranging from 25.0 to 0.32 mg./ml. in propylene glycol.

4. Combinations of penicillin and streptomycin under in vivo conditions do not destroy the enterococci.

5. Chloramphenicol should be added to the mixture of antibiotics to destroy the enterococci in the root canal. It also has many other desirable characteristics.

6. An antifungal antibiotic which can destroy the fungi present in the infected root canals is not available at the present time. A chemical fungicide must be added.

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# THE LACK OF ANTIVITAMIN ACTIVITY IN A HOMOLOGUE OF ASCORBIC ACID

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## INTRODUCTION

THE existence of substances which have come to be known as antivitamins has been recognized comparatively recently. Some of these substances are naturally occurring while others are synthetic. However, they all are said to act in a similar manner: by an interference with the synthesis or metabolism of a vitamin by (1) inactivation or chemical destruction, (2) irreversible combination, or (3) competitive inhibition.[1] This physiological interference can occur because of the close structural similarity between the vitamin itself and the corresponding antivitamin.

Organic chemists recognize several classes of structural similarities; one well-known class usually called "the homologous series" contains groups of compounds in which contiguous members differ by one $CH_2$ group. In the last few years it has been shown that some of these vitamin-like homologous compounds when fed to animals can produce signs which are suggestive of those produced by deficiencies of the various vitamins themselves. Similar conditions have also been seen in human beings. The classical antagonism between sulfanilamide and p-aminobenzoic acid was one of the first to be thoroughly established,[2] and since 1940 substances with antivitamin activity have been described in relation to many of the other vitamins. Thus, the following vitamins may be listed with an example of an antagonist: nicotinamide—pyridine-3-sulphonic acid,[3] thiamine—pyrithiamine,[4] riboflavin—lumiflavin,[5] vitamin E—$\alpha$-tocopherol quinone,[6] vitamin K—dicoumarin,[7] biotin—avidin,[8] pyridoxine—2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine,[9] inositol—$\gamma$-hexachlorocyclohexane,[10] and vitamin C—d-glucoascorbic acid.[11, 12]

Woolley and Krampitz[11] reported the production of a scurvylike condition in mice and cotton rats by feeding d-glucoascorbic acid (2,3-enediol-d-glucoheptono-1,4-lactone) (Fig. 1). The signs of this condition were diarrhea, extensive subcutaneous hemorrhages including hemorrhages in the gingivae, edema of the legs, wrists, and knees, a fiery red appearance of the lungs, joints of the legs, and ribs, and occasional alopecia, but no loosening of the teeth; the disease could not be prevented or cured by ascorbic acid. However, no microscopic studies of these animals were reported. Woolley[12] later fed this compound to guinea pigs and observed a condition which ascorbic acid could prevent under certain circumstances and concluded that this must be regarded as a true ascorbic acid deficiency, although again no changes were observed in the teeth.

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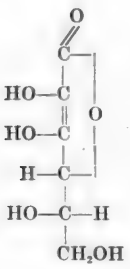
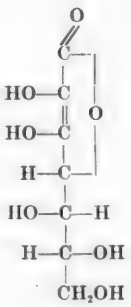
Banerjee and Elvehjem[13] also studied the effect of this compound on white rats, chicks, and guinea pigs and concluded from their work that the effect of d-glucoascorbic acid was not due entirely to an antivitamin C action but partly to a lack of some other factor supplied by the intestinal flora and present in liver powder.

Gould[14] more recently found no antagonistic action between d-glucoascorbic acid and ascorbic acid as judged by histological examination of the jaws of guinea pigs or by estimation of serum phosphatase levels in animals fed varying amounts of d-glucoascorbic acid.



ascorbic acid
(Vitamin C)



d-glucoascorbic acid
(a homologue of Vitamin C)

Fig. 1

The close relationship between scurvy and periodontal disease has been recognized for many years and the oral signs of vitamin C deficiency are too well known to discuss here. However, even today, with complete knowledge and material readily and cheaply available for prevention of the disease, scurvy is still present in this country.[15] With the more recent discovery of the role of ascorbic acid in wound healing,[16] along with the demonstration that certain infections and pathological conditions (e.g., tuberculosis,[17] hyperthyroidism,[18] and Addison's disease[19]) increase the daily requirement of ascorbic acid, the importance of this vitamin in the maintenance of oral and systemic health is increasingly being recognized. The steadily mounting number of synthetic drugs now available for therapeutic purposes raises the possibility that some compound, structurally related to ascorbic acid and acting as an antivitamin, may be formed in the body as a metabolic product. The previous studies with ascorbic acid homologues are not in agreement. Therefore, d-glucoascorbic acid was tested to determine whether a scorbutic condition could be induced in two animals not requiring dietary vitamin C, the rat and hamster, and in one animal requiring vitamin C, the guinea pig.

## EXPERIMENTAL OBSERVATIONS

Seven littermate albino rats were fed a natural ration while 7 rats from another litter received a purified synthetic diet, both studies beginning when the rats were 41 days of age. Five per cent of d-glucoascorbic acid was added to

the diet of 4 of the rats of the first litter while 10 per cent of the compound was incorporated in the diet of 4 rats from the second litter. By the end of the second day, all animals receiving glucoascorbic acid exhibited severe diarrhea, which continued throughout the experimental period. By the sixth day, alopecia of the neck could be seen in the experimental rats receiving the higher level of the compound. Roentgenograms of this litter, taken on the twelfth day, showed no detectable differences in density of cortical bone between control and experimental animals, while red blood cell count differences were also negative. Weight records of the animals of the second litter showed that the controls gained an average of 4.0 grams per day while the experimentals gained an average of only 0.7 gram per day (Fig. 2). All animals of both litters were sacrificed on the fourteenth and fifteenth days.

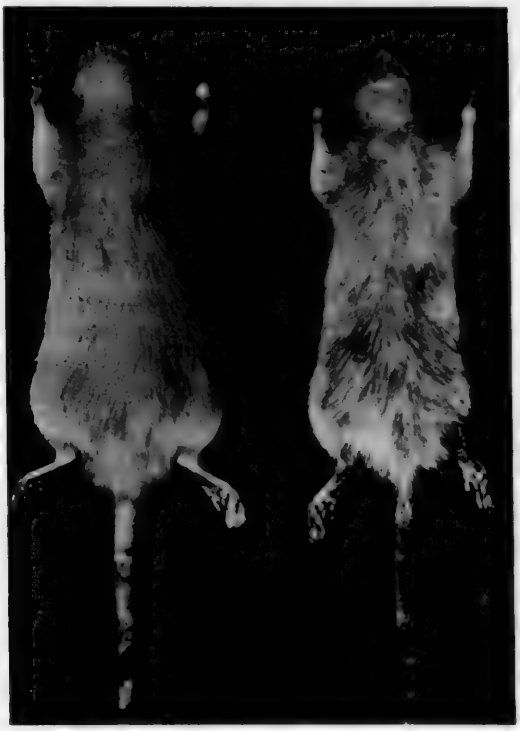


Fig. 2.—Littermate rats; animal on right received 10 per cent glucoascorbic acid in the diet. Note difference in size, alopecia of neck, and evidence of diarrhea on tail of experimental animal.

Gross autopsy findings were negative, and ascorbic acid determinations of livers and kidneys, by the indophenol method, also failed to show any significant differences. Histological sections of various organs of the animals receiving 10 per cent of the compound in the second litter were normal except for the livers of the experimental rats which exhibited a moderate degree of congestion and slight dilatation of the sinusoids. Sections of the knee joints through the femur

and tibia of the experimental animals in this same litter appeared to show a decrease in width of individual bony trabeculae in the diaphysis but no changes in the epiphyseal plate. Histological sections through the molars and incisors of these animals were normal except for a slight congestion in scattered areas of the periodontal membrane and pulp of the experimental group.

In testing the effect of this compound on the Syrian hamster, 2 litters (consisting of 7 and 8 animals, respectively) were given a purified synthetic diet beginning at 32 days of age. Ten per cent d-glucoascorbic acid was added to the diet of 4 animals of the first litter and 5 per cent of the compound was added to the diet of 5 animals of the second litter. The animals receiving either level of the compound developed severe diarrhea by the end of the second day as before. Within 4 days, all animals receiving 10 per cent of glucoascorbic acid were dead, while 4 of the 5 receiving 5 per cent died within 6 days. All experimental animals in this latter group showed a continual weight loss up to death (control hamsters gained an average of 2.0 grams per day; experimental hamsters lost an average of 1.3 grams per day).

Histological sections through the tibiae of the experimental animals receiving 5 per cent glucoascorbic acid showed a decrease in amount of bony trabeculae in the diaphysis, an apparent narrowing of the epiphyseal line (with more numerous degenerate cartilage cells), and marked congestion of red blood cells in the bone marrow. These changes in the bone are indicative of a decreased rate of growth and bear no resemblance to a typical scorbutic condition. Sections through lower incisors of exemplary control and experimental animals receiving the 5 per cent level showed a moderate degree of congestion in the pulp and periodontal membrane of the latter group but no changes in odontoblasts.

In addition, 1 per cent glucoascorbic acid was added to the same synthetic diet given 4 of 7 young adult hamsters; the remaining 3 animals served as controls. The 1 per cent addition was raised to 3 per cent after 7 days. Slight diarrhea was noted in these experimental animals only after 12 days. The animals in this group receiving the test compound died or were sacrificed between the fifteenth and twenty-fifth days.

Sections of the knee joints of these animals showed a greatly congested bone marrow but exhibited no other unusual findings. There were no changes in the dental structures of the animals receiving d-glucoascorbic acid for 1 to 3 weeks at the 3 per cent level.

In the final experiment, 12 guinea pigs were divided into 4 groups, each of which received a basic ration of finely ground Purina Laboratory Chow. Each animal of Group I received this diet and 5 mg. of ascorbic acid per day (per os by pipette). Group II received this diet with no additions or supplements. Group III received the basic diet with 5 per cent glucoascorbic acid added plus 5 mg. of ascorbic acid per day per animal. Group IV was treated the same as Group III except that the ascorbic acid was omitted. After the initiation of this diet, all animals thrived for about 2 weeks, then the control animals with-

out ascorbic acid (Group II) began to lose weight rapidly and died on the twenty-fourth and twenty-fifth days. The animals in the remaining groups, in apparently good health, were sacrificed at this time.

At autopsy, gross pathological lesions were absent except in the scorbutic animals (Group II) where hemorrhages about the knee joints were evident. In all groups, histological sections were prepared of the knee joint, lower incisor, and molars of each animal and the findings are described below.

*Group I. Guinea Pigs Which Received a Scorbutic Diet With Supplements of Ascorbic Acid*

*Knee joints.*—The epiphyseal line of the proximal end of the tibae showed orderly columns of cartilage cells, the usual amount of trabeculation in the diaphysis, and normal cellular elements in the bone marrow.

*Teeth.*—The incisor pulps showed the usual distribution of fibroblasts and blood vessels and a normal odontoblastic layer. The dentin and predentin layers were smooth and regular. The periodontal membrane was intact, although some blood vessels appeared slightly congested. The pulps of the molars showed a healthy layer of odontoblasts and the usual distribution of structural elements. The interdental bone was high and evenly rounded.

*Group II. Guinea Pigs Which Received no Supplements to the Scorbutic Diet*

*Knee joints.*—The cartilage cells of the epiphyseal line were poorly organized, atrophic, and not arranged in definite, regular columns. The zone of lacunar enlargement was conspicuously diminished and even absent in some areas. The epiphyseal line appeared narrow as contrasted to the Group I animals. There was a large amount of bony trabeculation on the diaphyseal side of the epiphysis, but the bone had a "moth-eaten" appearance, although no osteoclasts were evident. A large amount of fibrous connective tissue had invaded the marrow spaces with a corresponding decrease in cellular elements. The cortical bone appeared thinner in this group and subperiosteal and intramuscular hemorrhages were present in many areas.

*Teeth.*—The incisors showed a complete loss of odontoblasts and there were many free blood cells in the pulp. Pulpal to the dentin was a light staining zone, equal in width to the dentin, with a heterogeneous appearance, resembling "osteoid-matrix." The pulp appeared to consist of fewer cells and of more colloidal-like background substance. The periodontal membrane also contained many free red blood cells. The pulps of the molars were closed nearly to the basal end of the tooth by an irregular type of dentin. No odontoblasts were present. There were many areas of hemorrhage in both the pulp and periodontal membrane. The interdental bone consisted of only a few spicules which were very limited in height.

*Group III. Guinea Pigs Which Received Supplements of Ascorbic Acid and Glucoascorbic Acid to the Scorbutic Diet*

The sections of knee joints and teeth of the animals in this group were indistinguishable from those of the group receiving ascorbic acid but no glucoascorbic acid.

*Group IV. Guinea Pigs Which Received the Scorbutic Diet With Supplements of Glucoascorbic Acid*

*Knee joints.*—The cartilage columns of the epiphyseal plate were regular, the cartilage cells well defined and healthy. Trabeculation varied from slight to heavy but the bone was well rounded, and appeared perfectly normal. No fibrous tissue was present in the diaphysis. No hemorrhages were seen in this group.

*Teeth.*—In some areas, the layer of odontoblasts in the incisor pulps was distinct and quite intact. In other areas, the odontoblasts were entirely absent. Some congestion of blood vessels in the pulp could be seen. A rather wide zone of atypical dentin resembling a light staining heterogeneous matrix was apparent. Some sections showed evidence that the pulp was being rapidly filled in toward the incisal portion with a material greatly resembling bone, but even here there were some remnants of odontoblasts which appeared to be functional. The periodontal membrane was essentially normal. The pulps of the molars showed some irregular deposition of this bonelike material. However, odontoblasts were still present and, toward the basal end of the teeth, appeared completely unaffected. Hemorrhage was not present although congestion was prominent. The height of the interdental bone compared favorably with that of the normal animals.

## DISCUSSION

From the results obtained in those species of animals which do not require a dietary source of vitamin C, viz., the albino rat and Syrian hamster, the feeding of d-glucoascorbic acid failed to produce lesions which, by histological technic, could be diagnosed as scurvy.

The only gross pathologic signs seen in these animals were diarrhea, decreased rate of growth, and occasional alopecia. In no case was loosening of the teeth or bleeding of the gingivae observed. There were no significant differences in the red blood cell counts between rats receiving a plain synthetic diet and one containing 10 per cent glucoascorbic acid. By the indophenol test, no significant differences in ascorbic acid content of the livers and kidneys of these groups of animals were found.

By histologic means, it was possible to demonstrate a moderate degree of congestion in the liver, kidney, bone marrow, and dental pulps in some of the rats and hamsters receiving glucoascorbic acid. There appeared to be some differences in the sections of tibae, more suggestive of a decreased rate of growth than of a true scorbutic condition. Although many sections of the jaws of these animals were prepared, in no case could any deviation from the normal structure be observed except for the moderate congestion seen in the pulp and periodontal membrane. Since it has been established that the dental apparatus is the first of the body structures to show pathologic changes with the onset of a scorbutic state, it must be concluded that d-glucoascorbic acid is not capable of inducing scurvy in the rat and hamster, two animals not requiring dietary vitamin C.

In the guinea pig, an animal which requires dietary vitamin C, the results again lead to the conclusion that the compound under study does not possess

antivitamin C activity. The group of animals receiving the vitamin C-deficient stock diet with daily supplements of ascorbic acid grew normally and showed no pathologic signs, either grossly or histologically, at the time of sacrifice. The guinea pigs receiving the diet without ascorbic acid supplements developed a typical scorbutic condition, as expected.

The most interesting results were obtained in the two groups receiving glucoascorbic acid, with and without ascorbic acid. The group which received ascorbic and glucoascorbic acid showed no histological abnormalities. There were no subcutaneous or intramuscular hemorrhages, the epiphyseal plate in all cases was orderly and regular, trabeculation was prominent and of a normal character, and there was no invasion of the diaphysis with the fibrous connective tissue seen in the scorbutic animals. The sections through the incisors and molars of this group of animals showed perfectly normal structures. There were no disturbances in the pulp or odontoblasts and the sections compared favorably with those of the animals receiving ascorbic acid but no glucoascorbic acid. Evidently, the simultaneous administration of the vitamin and the homologue in no way interfered with the normal utilization of the vitamin.

The sections from animals receiving glucoascorbic acid but no ascorbic acid showed consistent findings. The histological picture of the knee joints was that of a perfectly normal animal. If a diagnosis were to be made on the basis of the knee joints alone, it would be necessary to say that scurvy was entirely absent. However, sections of the incisors and molars indicated differently. Although the complete disappearance of odontoblasts as noted in the scorbutic control group was not seen, the odontoblasts were absent in some areas and appeared degenerate in others. The deposition of irregular dentin appeared to be of the same type as that seen in the scorbutic group. From the over-all picture of this group of animals receiving only glucoascorbic acid in the diet, there is evidence either of a lag period in the development of the scorbutic condition or a lessening in the intensity of the condition. In either case, it appears likely that rather than inhibiting the protective action of ascorbic acid, there may be even some Vitamin C activity of the compound in the absence of ascorbic acid, although, of course, complete protection against scurvy is lacking.

These results are at variance with those of Woolley who reported evidence of an antivitamin activity. This difference in findings may be due in part to the use of histologic sections in the current studies for the diagnosis of scurvy instead of reliance on gross signs.

## SUMMARY AND CONCLUSIONS

d-Glucoascorbic acid in varying concentrations was fed to groups of rats, hamsters, and guinea pigs, and, although some systemic reactions were noted, no signs of scurvy developed which could be demonstrated by histological means. The systemic reactions may have been due to a toxic action of the compound. In the guinea pig, it appears that the feeding of d-glucoascorbic acid may give a slight protective action against scurvy, but it will not com-

pletely prevent the disease. From the evidence presented, the hypothesis of a competitive or inhibitive role of d-glucoascorbic acid against vitamin C is untenable.

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# THE EFFECT OF SINGLE DOSES OF IRRADIATION UPON THE ERUPTION OF THE UPPER RAT INCISOR

H. MEDAK, I. SCHOUR, AND W. A. KLAUBER, JR.

*From the Department of Histology, College of Dentistry, and Department of Radiology, College of Medicine, University of Illinois, Chicago, Ill.*

THE purpose of this study was to evaluate quantitatively the effects of irradiation upon tooth eruption. The incisor of the rat promised to be a valuable test object. This tooth undergoes continuous and relatively rapid growth and eruption. Its basal portion consists of epithelial and connective tissue cells which proliferate and differentiate continuously. These cells could be expected to be more susceptible to x-ray irradiation than mature cells.[1]

Direct measurements of the rate of eruption of irradiated incisors of the rat have not been reported. Leist[2, 3] and Burstone[4] concluded from histologic studies that the rate of eruption of the rat incisor is retarded following x-ray irradiation.

## MATERIALS AND METHODS

Eighty-four albino rats, ranging in age from 30 to 90 days and weighing from 80 to 200 grams at the beginning of the experiment, were studied. Groups of 8 animals were exposed to 500 r., 1000 r., 2000 r., and 2500 r., in air, respectively. Twelve rats received 3000 r., and 28 received 4000 r. Twelve rats were used as controls (Table I). Histologic studies of the material are in progress and the findings will be reported at a later date.

TABLE I

NUMBER OF RATS, DOSAGE OF RADIATION IN ROENTGEN UNITS, AND SURVIVAL TIME IN WEEKS

| DOSE | NO. OF ANIMALS | SURVIVAL TIME (WEEKS) | | | | | | | SURVIVAL OVER 20 WEEKS |
|---|---|---|---|---|---|---|---|---|---|
| | | 0-2 | 3-4 | 5-6 | 7-8 | 9-10 | 11-12 | 13-16 | |
| 500 r. | 8 | – | 2 | – | 1 | – | – | 1 | 4 |
| 1000 r. | 8 | 1 | 1 | – | – | – | – | 2 | 4 |
| 2000 r. | 8 | 2 | 1 | – | – | – | – | – | 5 |
| 2500 r. | 8 | 1 | – | 2 | – | 2 | – | – | 3 |
| 3000 r. | 12 | – | 4 | 1 | 2 | 1 | 1 | – | 3 |
| 4000 r. | 28 | 11 | 5 | 3 | 2 | 1 | 1 | 1 | 4 |
| Controls | 12 | – | – | – | – | 1 | 1 | – | 10 |

The apparatus used in our experiment was a Keleket Therapy Machine, mobile unit (up to 140 kv.). The physical factors used were: 100 kv., H.V.L. of 1 mm. Al. (inherent filtration), 5 Ma., 12½ cm. t.s.d., a field of 2.5 cm. diameter; no additional filter was used. The output was 320 r. per minute in air. The anesthetized animal was placed on a treatment table of soft pine wood covered with 2 cm. of cotton batting and leatherette. The element of back scatter proved to be negligible upon comparison of measurements in air with measurements taken with the ionization chamber in place of the animal. The

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only variable was the length of exposure. Each animal received a single dose of irradiation. Prior to irradiation the rats were anesthetized intraperitoneally with 0.3 to 0.5 cm. of a 1 per cent solution of Nembutal. The body was covered with lead plates, leaving the anterior part of the head exposed. Care was taken in positioning to direct the central rays toward the basal end of the upper incisor. A record of the exposed area was obtained by taking a roentgenogram immediately before the experimental irradiation. The weekly measurements for the right and left incisors were recorded on graphs. The line of regression for each group of animals was determined by the method of the least squares. The results are shown in Fig. 1.

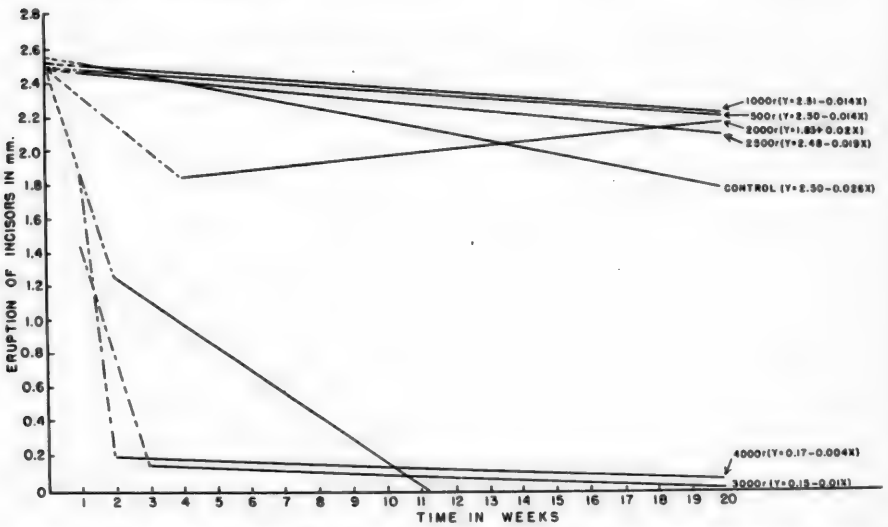


Fig. 1.—The rate of eruption of upper incisor of albino rat after irradiation with a single dose varying from 500 to 4000 r. Each line represents the line of regression ($y = a + bx$). Y represents the amount of eruption in mm. per week; x, the number of weeks intervening between irradiation and termination of experiment; b, the acceleration or decelaration in eruption (the weekly rate of increase or decrease in mm.) and a, indicates the origin of the line.

At the beginning of the experiment the majority of the rats receiving 3000 and 4000 r. died between the twelfth and fifteenth day following irradiation with forced respiration and marked distention of the abdomen. This was probably caused by nasal occlusion. The high position of the laryngeal entrance in the nasopharynx above the palatopharyngeal folds does not permit the animal to live by mouthbreathing alone. Administration of daily doses of 3000 to 6000 units of penicillin, removal of the crustations from the nares which developed at the end of the second week, and insertion of a fine plastic tube into the nostrils, to keep the air passages open, reduced the death rate considerably.

## RESULTS

*Changes in the Skin.*—The group of rats receiving 500 r. did not show any changes in the skin. Rats receiving 1000 r. developed a slight erythema in the exposed areas, on or about the tenth day. Rats irradiated with 2000 r. showed,

in addition to the erythema, slight exudation on the palpebral borders of the eyes and epilation from the irradiated skin. The hair started to regenerate during the fifth or sixth week, but it was fuzzier and less dense. Irradiation with 3000 r. and 4000 r. produced reddening and exudation of the palpebral borders after one week. At the end of the second week, the orbital tissues were swollen and infected, leading to complete closure of the eyes by the third week and to peribulbar abscesses. In some cases necrosis and exfoliation of the eyes were seen. The hair did not regenerate in the epilated areas. A brownish exudate was observed in the oral cavity and the nostrils were closed by crusting.

*Eruption.*—A straight line of regression was derived from the data, using the method of the least squares for each group of animals irradiated (Fig. 1).

In the control animals the weekly rate of eruption was 2.6 ± 0.6 mm. and the rate of eruption decreased 0.026 mm. per week. Our findings of 2.6 mm. per week for the controls, rather than the generally accepted 2.1 mm. per week, might be due to the use of young animals in our experiment. The effect on the rate of eruption in the experimental animals depended primarily on the amount of irradiation given. Animals receiving 500 and 1000 r. showed no retardation in eruption, but rather a slight increase in rate. Disturbance in the rate of eruption was first noticed in the animals irradiated with 2000 r. Eruption was decreased during the first 4 weeks and then increased gradually to the level of the animals irradiated with lower doses. The animals which received 2500 r. presented 2 groups with such clearly divided results that it was necessary to graph them in 2 lines. One group showed slight stimulation as did the animals receiving 1000 r. The other showed a deceleration and cessation of eruption similar to the animals receiving 3000 r. Irradiation with 3000 and 4000 r. produced cessation of eruption within 3 weeks. Therefore, 3000 r. represents the minimal effective dose for the retardation of eruption with a critical point at 2500 r.

## DISCUSSION

The findings showed that the irradiation of the incisor area of the rat retarded and in higher doses stopped the eruption of the tooth. However, the experiments do not present a clear answer to the question whether or not the influence of eruption is directly through local injury of the odontogenic area or is induced by injury of the hypophysis. Such damage in turn could be caused either by primary or secondary irradiation of the pituitary gland. That the hypophysis plays an important role in tooth eruption has been shown conclusively by Schour and Van Dyke,[5] and Becks, Collins, Simpson, and Evans.[6] In order to test the possible role of this gland in our experimental results, the pituitary gland was irradiated in 10 rats with 4000 r. under the same standard conditions as used in the previous experiments. By taking roentgenograms prior to treatment with the animal and apparatus in position, it could be shown that the base of the upper rat incisor was outside of the field of irradiation. Measurements in the 5 animals surviving longer than 2 weeks revealed that the eruption of the upper incisor was not retarded. We, therefore, concluded

that the retardation and complete inhibition of eruption in our experiment was caused by the direct effect of the x-rays on the growing tissues of the incisor rather than by injury of the pituitary gland.

## SUMMARY

1. The rate of eruption of the upper incisors was measured in 84 albino rats ranging in age from 30 to 90 days following a single dose of x-ray irradiation, which was directed to the base of the upper incisors. The radiation dose ranged from 500 to 4000 r. in air. The rate of eruption was also measured in 12 normal white rats of similar age which were not exposed to irradiation.

2. The animals receiving 2000 and 2500 r. could be divided into 2 groups. One showed an increase, the other showed deceleration and cessation of eruption. Animals receiving 3000 and 4000 r. showed a depression in the rate of eruption within the first 2 weeks and then complete cessation.

3. Irradiation of the region of the hypophysis did not affect the rate of eruption.

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## CORRESPONDENCE

### AMMONIUM IONS VS. ORAL LACTOBACILLI

July 13, 1950

Dr. Hamilton B. G. Robinson
Ohio State University
Columbus 10, Ohio

Dear Editor:

Your June, 1950, issue of the *Journal of Dental Research* contained an article on page 320 reporting the failure of ammonium ions to inhibit oral lactobacilli. The experiments appear to have been meticulously conducted and the procedures carefully planned. The results disagree with findings previously reported by us and the inference is that our findings are not supported.

In fairness to our earlier work it should be pointed out that studies conducted by Doctors Kirchheimer and Douglas did not duplicate our procedures in several important respects.

1. The inoculum used by Kirchheimer and Douglas was 0.1 ml. of a 24-hour culture of the test organism. As reported, our studies were done with 0.1 ml. of a 1:100 dilution of a 24-hour culture. Hence, Kirchheimer and Douglas used inoculum containing one hundred times as many organisms. In testing the effectiveness of an antibacterial agent, the amount of inoculum is an important consideration.

2. Our studies of the possible inhibitory effectiveness of ammonia were based on observations of the ammonia content in stagnating saliva. These ammonia levels were determined by measuring the ammonia nitrogen content, not the ammonium salt content. Consequently, our testing of the effectiveness of various ammonium salts was based on the amount of ammonium nitrogen the salt supplied the testing medium and not on the amount of the ammonium salt. For instance, ammonium carbonate contains 37.4 per cent by weight of ammonium nitrogen, therefore 1 mg. of ammonium carbonate would provide only 37.4 per cent of the ammonia nitrogen we recommended.

3. A discrepancy appears on page 322. In the text the statement is made: "Duplicate sets of ten tubes of Medium 1 containing ammonium carbonate in amounts ranging from 0.56 to 5.6 mg. per tube, and control tubes of Medium 1 containing no ammonium carbonate, were inoculated with 0.1 ml. amounts of 24-hour cultures of the respective organisms." Table II gives the ammonium carbonate content of the tubes as 5.6 to 56.0 mg. per tube. We don't know which actually was employed but even if the larger amount were used, we are not surprised at the failure to get inhibition because of the heavy inoculum and the reduced ammonia nitrogen.

Kirchheimer and Douglas have not demonstrated that ammonia will not inhibit various strains of oral lactobacilli under conditions comparable to those used by us.

Sincerely yours,
Robert G. Kesel
College of Dentistry
University of Illinois
Chicago, Ill.

---

August 2, 1950

Dr. Hamilton B. G. Robinson
Journal of Dental Research
Ohio State University School of
Dentistry
Columbus 10, Ohio

Dear Dr. Robinson:

Thank you for forwarding a copy of the letter written to you by Dr. Robert G. Kesel on July 13, 1950.

In point 1 of his letter Dr. Kesel points out that the discrepancies between his and our findings might have arisen from the fact that in our experiments much larger inocula were used. In paragraph 3, page 321, of our paper we stated that "The amount of inoculum used had no effect upon the outcome of the experiments. Identical results were obtained whether the inoculum consisted of 0.1 ml. or a loopful per tube." Our standard loops deliver approximately 4 mg., or 0.004 ml.

In point 2 of Kesel's letter the statement is made that the inhibitory effectiveness of ammonia was not based on ammonium salt content, but rather on ammonia nitrogen content. Concerning this statement we would like to quote from the paper of Kesel and associates. *Am. J. Orthodontics* **33**: 87, 1947, paragraph 1, "Varying amounts of ammonium carbonate were added to tubes of sterile broth medium and these combinations were tested for their lactobacillus-inhibitory potency by the usual method." The results of this experiment are shown in Fig. 5 on pages 90 and 91. The legend below the photographs on page 90 states in effect that volumes ranging from 0.1 to 0.5 ml. of an ammonium carbonate solution containing 5.6 mg. of ammonium carbonate per ml. were used in these experiments. The photographs show that as little as 0.2 ml. of this solution was bactericidal within 24 hours. In paragraph 2 on page 87 experiments are described in which various amounts of an ammonium acetate solution containing 5.6 mg. per milliliter were added to test tubes of broth medium. These tubes then were inoculated with lactobacilli. There was complete inhibition of growth in the tubes which had received 0.5 and 1.0 ml., respectively, of the ammonium acetate solution.

Point 3 seems to have arisen because of failure of Dr. Kesel to read the three lines of text found below Table II on page 322 of our paper.

We also would like to point out that Kesel and associates in their publication failed to indicate the size of the bacterial inoculum used in the experiments quoted above. On page 83 of Kesel's paper, reference is made to the bacterial concentration in the "culture filtrate." "The concentration of the culture in the filtrate was 1:100." In his letter, however, Kesel states that, "As reported, our studies were done with 0.1 ml. of a 1:100 dilution of a twenty-four-hour culture."

Additional evidence that ammonium ions per se do not exert a bactericidal effect upon lactic acid bacteria will be found in the paper of Jenkins et al., *Nature* **105**: 606, 1950. Pearlman (*J. D. Res.* **28**: 638, 1949) and Stephan (*J. D. Res.* **28**: 652, 1949) reported that they failed to notice significant differences in the effect of sodium potassium and ammonium ions on lactobacilli.

Sincerely yours,
Howard C. Douglas
Waldemar F. Kirchheimer
The Medical School
Northwestern University
Chicago, Ill.

## ERRATUM

In the paper by Marcu Brucker: Studies on the Incidence and Cause of Dental Defects in Children, IX. The Deciduous Molars: Fissures and Caries, *J. D. Res.* **29**: 325-330, 1950, the words LEFT and RIGHT under LOWER MOLARS in Table IV, page 327, should be transposed.

In the paper by Loren W. Gruber: Preliminary Report on the Use of Xylocaine as a Local Anesthetic in Dentistry, *J. D. Res.* **29**: 137-142, 1950, the fourth paragraph on page 137 should read "In the course of study of Xylocaine, Gordh has noticed only two cases of toxic reactions after deliberate overdosing (1.35 and 3.0 Gm., respectively, as compared with 40 mg. used in dentistry)." In the original 4 mg. inadvertently appeared instead of 40 mg.

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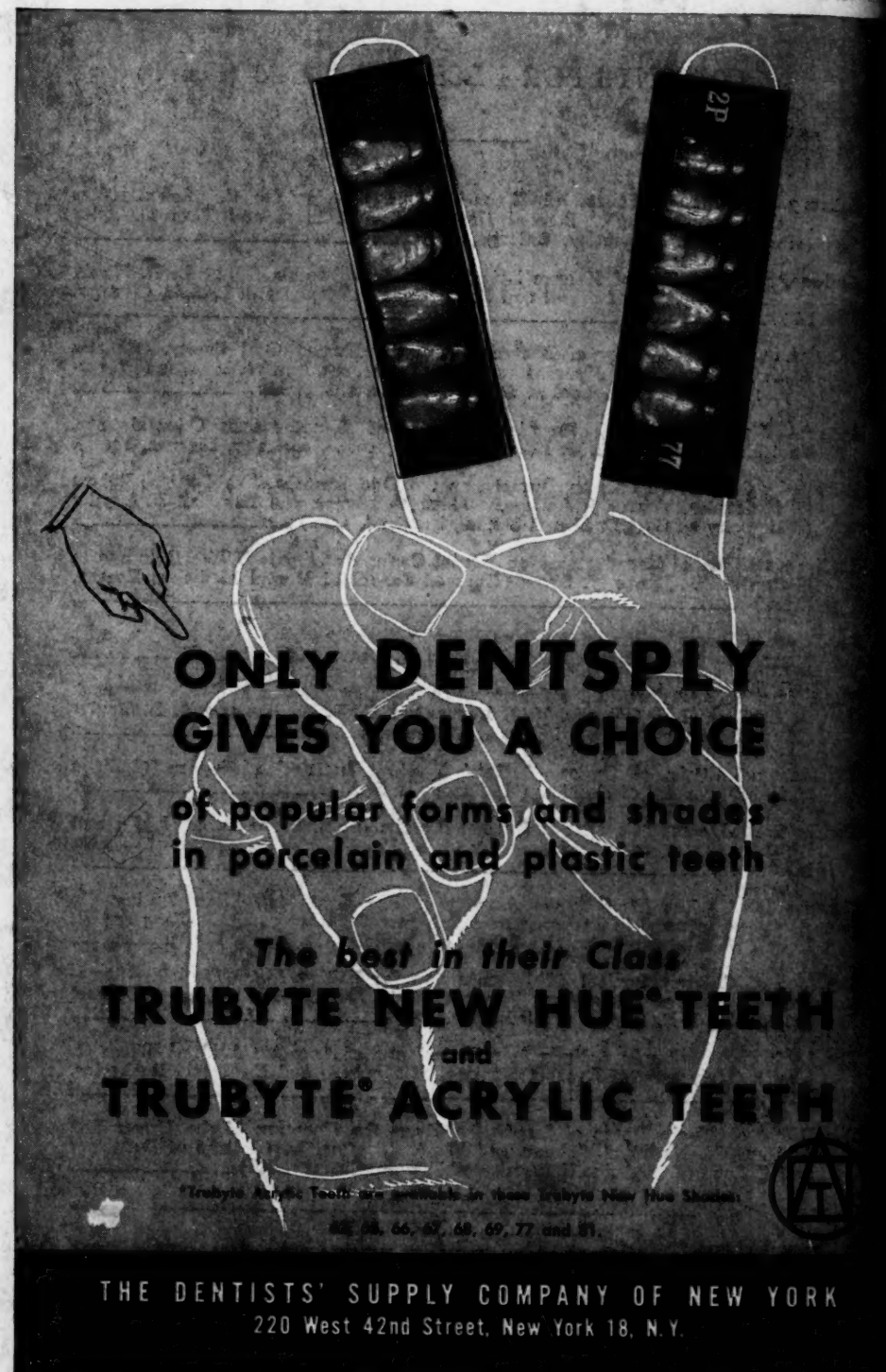


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